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II-1 The importance of the outer mitochondrial compartment in regulation of energy metabolism

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Abstract

Substitution of physiologically present macromolecules during isolation of mitochondria and investigation of their functions led to a significant change in regulation of oxidative phosphorylation. The differences compared to conventionally isolated mitochondria were that stimulation of oxidative phosphorylation appeared to rather depend on the activity of peripheral kinases than on the addition of free ADP. The localisation of peripheral kinases such as hexokinase and mitochondrial creatine kinase are described as well as the effects of macromolecules on the regulation of bound hexokinase and of oxidative phosphorylation via this enzyme. (Mol Cell Biochem **133/134:** 69–83, 1994)

Key words: mitochondria, brain, liver, macromolecules, localisation, hexokinase, mitochondrial creatine kinase, oxidative phosphorylation

Introduction

Different levels of muscle function and mitochondrial regulation

Muscle cells are excitable as nerve cells, and have a highly specialised contractile apparatus which relates to the cytoskeleton of other cells. In addition skeletal muscle cells resemble hepatocytes as they take up glucose to form glycogen- and amino acids to build proteins stores. All these processes depend directly or indirectly on ATP, provided mainly by oxidative phosphorylation. Membrane depolarisation and repolarisation during excitation, muscle contraction and relaxation as well as Ca^{2+} release and sequestration are processes with a time scale of milliseconds while the synthesis of glycogen and proteins proceeds in seconds. To avoid large ATP/ADP fluctuations during the former fast and energy consuming processes creatine/phosphocreatine is used as an energy buffer and energy transferring system. By this way, high ATP turnover during excitation increases ADP levels only locally but the creatine level (instead of ADP) globally. The latter would then serve as a signal to activate mitochondrial metabolism. We hypothesise that during rest, metabolites other than free ADP also regulate the mitochondrial activity. When oxidative phosphorylation generates the energy needed for building up glycogen and protein stores, the mitochondrial metabolism may be activated by metabolites which are utilised in these pathways, such as glucose, glycerol, UDP and GDP. The activation is performed via specific kinases which are organised at the mitochondrial surface and directly communicate with the inner mitochondrial compartment. This mitochondrial coupling of kinases, such

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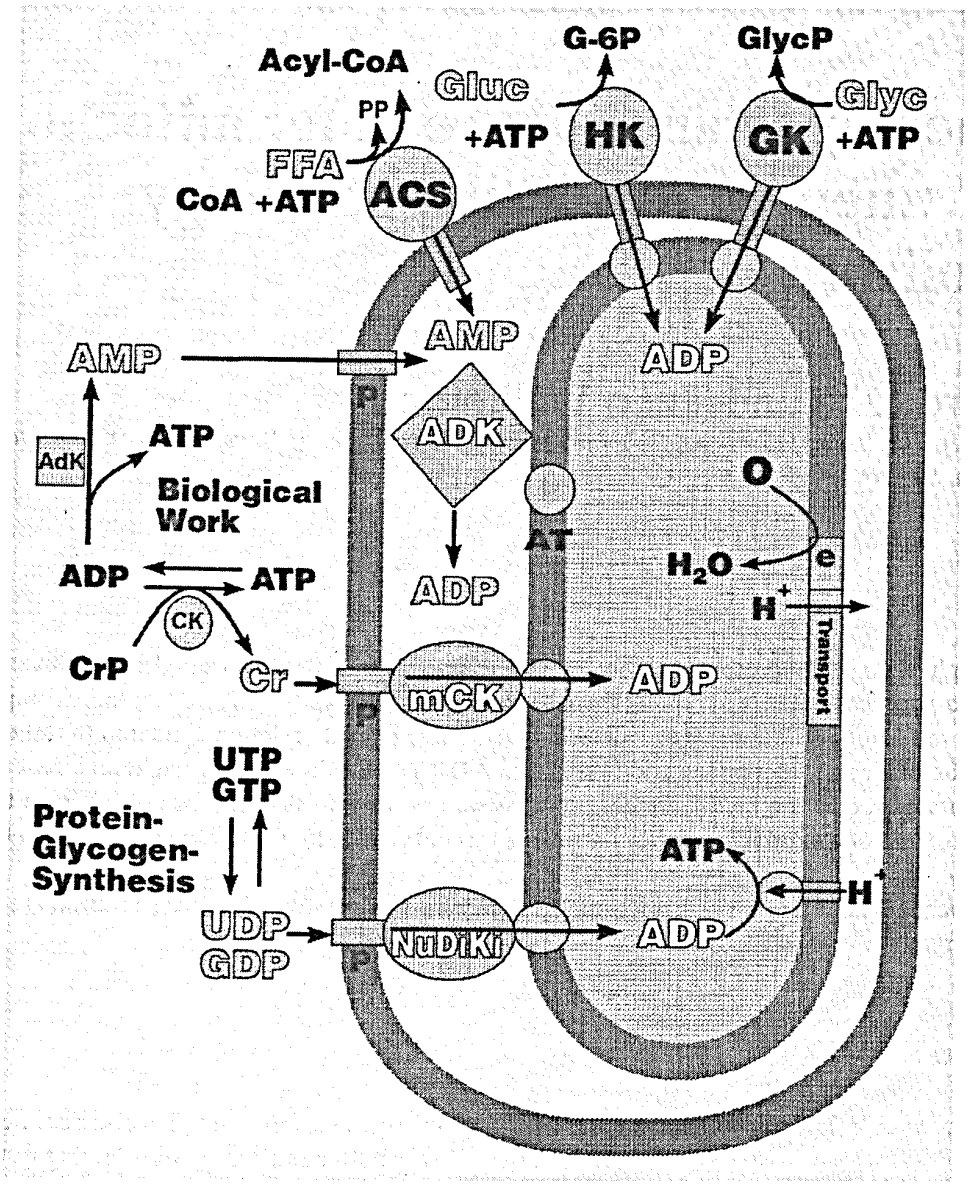


Fig. 1. Scheme showing the organisation and function of several kinases at the mitochondrial surface. On group of kinases named 'energy consuming kinases' is bound to the outer membrane pore protein: acyl-CoA synthase (ACS), hexokinase (HK), and glycerol kinase (GK). A second group named 'energy transferring' kinases is located between the two envelope membranes: adenylate kinase (ADK), mitochondrial creatine kinase (mCK), nucleoside-diphosphate kinase (NuDiKi). Some kinases (HK, GK, mCK, NuDiKi), which were enriched in the contact site fraction, are thought to form complexes with the outer membrane pore (P) and the adenine nucleotide translocator (AT). To keep the scheme clear, the reactions of several kinases are shown incompletely in abbreviated form.

as known for hexokinase, may accomplish an additional function in permanently working muscles like heart or in nerve cells which depend more or less on blood born substrates, it may regulate the cellular substrate (glucose) uptake according to the mitochondrial activity. In summary, the coupling of kinases to the inner compartment serves to transmit information in both directions either about increased ATP turnover and/or extramitochondrial substrate level directed towards the mitochondria or about mitochondrial activity directed towards the substrate supplying metabolic pathways.

Specific organisation of kinases at the mitochondrial surface

Two groups of kinases are found at the mitochondrial surface (Fig. 1). **Energy consuming kinases** such as acyl-

CoA-synthase (ACS), hexokinase (HK) and glycerolkinase (GK) of which the latter two specifically bind to the outer membrane pore protein [1, 2]. A second group, for example adenylate kinase (ADK) creatine kinase (CK) and nucleoside diphosphate kinase (NuDiKi), is located between the two envelope membranes. These kinases are called **energy transmitting kinases** because their products are energy rich and able to form ATP.

During kinetic analyses these kinases appeared to be functionally coupled to the inner mitochondrial ATP [1, 3–9], meaning that they interact directly with the adenine nucleotide translocator in the inner membrane. The structural basis of this coupling may be the contact sites between the two mitochondrial boundary membranes in which the kinases may form complexes with the adenylate translocator and the outer membrane pore [10]. During preparation and characterisation of contact sites it was learned that the structure of these complexes was effected by conventional isolation in sucrose media.

Conventional isolation of the mitochondria destroys the structure of the outer compartment

The function of the outer mitochondrial compartment in regulation of mitochondrial metabolism has so far not been regarded. The reason for this blind spot in our knowledge was that this compartment is severely disintegrated during the standard isolation of mitochondria. The commonly used media adjust the physiological osmotic pressure by sugars such as mannitol or sucrose. Because exclusively the inner membrane is semipermeable for the sugar molecules, these media preserve the structure and function of the inner membrane matrix compartment. The structure of the outer mitochondrial compartment, however, is strongly altered during isolation as the approximately 30% solution of cytosolic proteins which do not permeate the outer membrane is completely removed. The structural changes caused by isolation compared to the mitochondrial morphology *in situ* are known since a long time [11]. They were visible as swelling of the intercristae space and the space between the two envelope membranes. However, specific functions of the intact structure serving to estimate the role of the outer compartment were missing completely. We supposed that contact sites between the two boundary membranes represent reminiscent structures of the physiological organisation of the outer mitochondrial compartment in intact mitochondria [10]. A function of contact sites in regulation of transport and exchange processes either of precursor proteins [12] or metabolites [13] has been shown, which emphasises the importance of the outer compartment in regulation of mitochondrial activity.

These considerations led us try to restore an intact outer compartment by substituting the physiologically present proteins with dextrans [14, 15]. Based on the experience with contact sites, we were able to define several structural and functional criteria to prove the intactness of the outer compartment: i) narrow space between cristae and the two boundary membranes, ii) increase in contact site frequency in freeze fractured mitochondria, iii) changes in binding affinity of proteins to the mitochondrial surface, iv) changes in kinetic properties of enzymes located in the outer compartment, v) reduction of exchange rate of adenine nucleotides.

The experiments were designed in two ways: the structure of the outer compartment was either restored in conventionally isolated mitochondria by subsequent addition of macromolecules or the structure was preserved by the presence of macromolecules during isolation. By the first method, it was possible to study the effect of gradual restoration of the outer compartment, while the other method served to analyse physiological binding of proteins to the surface of mitochondria.

Results and discussion

Effect of macromolecules on the structure of the outer compartment

Conventionally isolated liver mitochondria were subjected to rapid freezing and freeze fracture (Fig. 2). The presence of 10% dextran Mr 70 kDa led to a significant reduction of the space between the two envelope- and the crista membranes. The cristae visible in cross fractures of control mitochondria were swollen and exhibited the same, pears like, shape as in thin sections, whereas they had a long, thin structure in dextran treated mitochondria. The close attachment of the envelope membranes under dextran suggested changes in protein organisation in the outer compartment and rose the question whether this would also affect contact formation.

We previously developed a method in freeze fractured mitochondria to estimate the frequency of contact sites [16]. In mitochondrial freeze fractures the fracture plane often jumps between the two boundary mem-

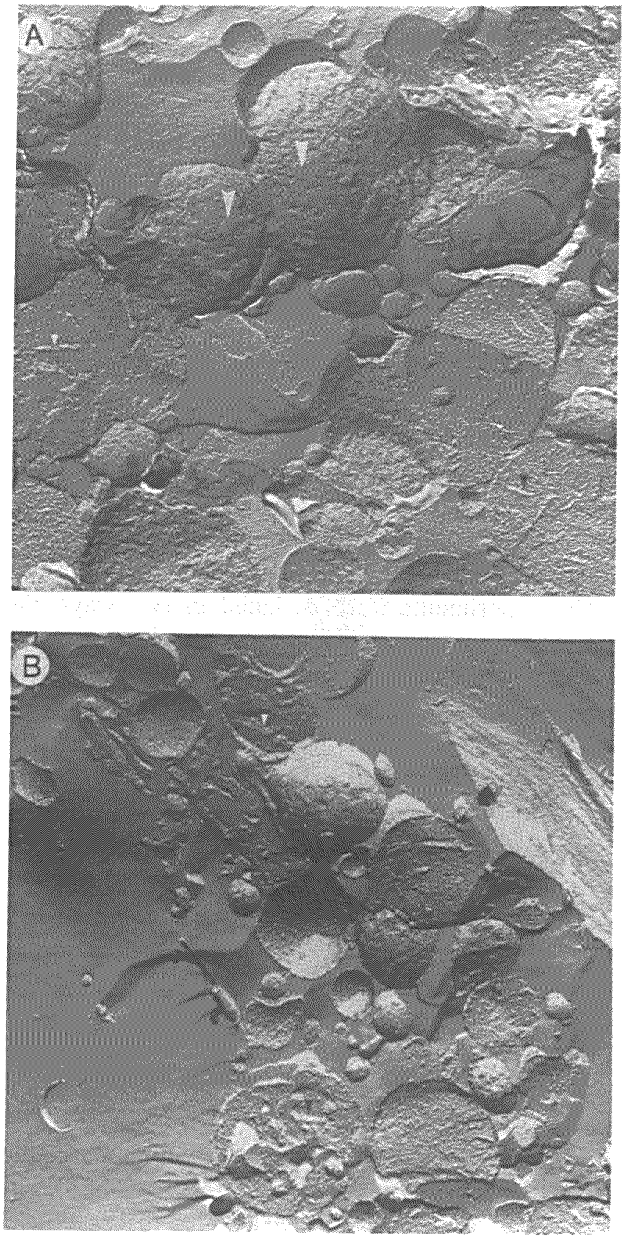


Fig. 2. Surveys of the isolated mitochondrial fraction from rat liver. (A) Mitochondria in the presence of 10% Dextran-70, (B) control mitochondria. The convex fractures faces show fracture plane deflections. Large, white arrowheads point to smooth patches of the exoplasmic face of the outer membrane laying on top of the particle rich protoplasmic face of the inner membrane. In contrast to the control, cross fractured mitochondria in the presence of Dextran-70 exhibit narrow crista like structures (small arrowheads) and no space between the two envelope membranes. Magnification 25,000. *Methods:* The mitochondrial samples were incubated in 113 mM sucrose, 10 mM K_2HPO_4 (pH 7.4), 5 mM $MgCl_2$, 12.5 mM succinate. The oxidation rate was stimulated by addition of 10 mM ADP (state 3). Mitochondria were directly subjected to rapid freezing by the sandwich technique as described by Knoll *et al.* [50]. The time to cryofixation was approximately 15 s. The samples were broken in a Balzers BAF 301 freeze etch device at – 100° C and (2–7) 10^{-7} Torr, followed by Pt/C and C shadowing. For electron microscopy, a Hitachi H7000 instrument at 75 KV was used. The nomenclature of the exposed membranes follows that of Branton *et al.* [51].

Table I. Regulation of contact sites and bound hexokinase activity in hepatocytes and isolated liver mitochondria

Effector	Relative contact site frequency	Relative bound hexokinase activity	Ref.
State 4 (O2, Pi, substrate present)	1	1	16, 17, 41
State 3 (O2, Pi, substrate, ADP present)	3.85	4–5	16, 17, 41
ADP (Pi lacking)	3.85	4–5	41
Atractyloside	3.85	nd	41
10% Dextran 70 state 4	3.70	nd	14
10% Dextran 70 state 3	6.15	6	14
DNP (uncoupler)	0.15	0.2	16, 23
20% Glycerol	0.12	nd	16
Fatty acids	1.35	0.24	17
Glucagon (β-receptor)	2.23	0.64	18
Epinephrine (α1-receptor)	6.54	nd	18

As a means of quantifying the difference in fracture-plane deflections, the length (L) of the edge where the fracture plane deflects was measured as it related to the corresponding mitochondrial area. In convex fractures, the length of the edge of the exoplasmic face of the outer membrane was measured, whereas, in concave fractures, the deflection line on the exoplasmic face of the inner membrane was determined. These measurements of L were expressed as length (μm) per unit of mitochondrial fractured membrane area. In every population of mitochondria, there are some which are completely void of fracture plane deflections. The number being dependent upon the metabolic state of the whole sample. To compensate for these differences we adjusted our calculation of fracture plane deflections by first determining on survey pictures the total area of mitochondria with no deflections. M_s and those with deflections M_p, and then normalising these values by the expression $M_p(M_p + M_s)$. The final value for quantification of freeze fracture deflections L_p was then calculated from the equation: L_p $(\mu m/\mu m^2) = L\, M_p(M_s + M_p)$, and the statistical differences obtained by applying the U-Test. The measurements were made in the areas where the curvature was low to avoid large distortions of the measured edge lines. The data in the Table are contact site frquency and bound hexokinase activity relative to the values observed in mitochondria in state 4 (set to 1). The data were taken from the quoted publications.

branes. As a result one observes for example in convex fractures shown in Fig. 2A (large arrowheads) a frequent change between the smooth inner leaflet (exoplasmic face) of the outer membrane and the particle rich inner leaflet (protoplasmic face) of the inner membrane. The contact site analysis was based on the assumption that the contact sites were responsible for the jumping of the fracture plane and that the frequency of fracture plane deflections between the two boundary membranes would therefore correlate with that of the contact sites. By determination of fracture plane deflec-

tions in different mitochondrial states it was observed that phosphorylating mitochondria in state 3 exhibited a 3 to 4 times higher frequency of contact sites than mitochondria in state 4 (Table 1). In the presence of 10% dextran Mr 70 kDa the frequency of fracture plane deflections was already higher in state 4 and comparable to that of control mitochondria in state 3, while full activation of mitochondrial respiration in state 3 led to a further increase of contacts (Table 1). On the whole, electron microscopy showed that dextran reduced the space between boundary and crista membranes and induced contact site formation.

Composition of the contact sites

The observation that the frequency of contact sites was dependent on the functional state of mitochondria pointed to a dynamic nature of these structures. Indeed the contacts appeared to be subjected to hormonal and metabolite control (Table 1). While glucagon and free fatty acids decreased, epinephrine and insulin increased the frequency of contacts in hepatocytes [17, 18]. In all cases of different contact frequency we met a positive correlation between contacts and the amount of surface bound hexokinase (see column 3 Table 1 and ref. 17–19).

Contact site analysis by electron microscopy

The distribution of hexokinase was therefore analysed by means of specific antibodies at the electron microscopic level, it was observed in intact liver and brain mitochondria that hexokinase was preferentially located in attachment points between the two boundary membranes [20, 21] (schematically shown in Fig. 3). Because of this location hexokinase is not removed by disruption of the outer membrane using digitonin which leaves the contacts intact. When liver mitochondria were treated with hexokinase antibodies and subsequently incubated with 100 µg digitonin, the gold grains, representing hexokinase, were found inside the outer membrane vesicles which remained attached to the inner boundary membrane (Fig. 4A–C and schematically Fig. 3) while the surface of the inner envelope membrane was not immuno-reactive.

Contact site analysis by isolation

Based on the electron microscopic observation we judged hexokinase as a specific marker enzyme of contacts, while attempting to isolate the contact sites from osmotically disrupted mitochondria. By sucrose gradient centrifugation of the membrane fragments we isolated from brain, kidney and liver mitochondria a fraction of intermediate density besides outer or inner membrane fractions. In this intermediate fraction hexokinase activity was concentrated, which led us to use hexokinase antibodies and precipitate the bound enzyme attempting to identify presumably associated components [22]. The precipitate derived from the contact site fraction of brain and kidney mitochondria contained activity of marker enzymes of outer- (monoamine oxidase) and inner membrane (succinate dehydrogenase) and in addition to hexokinase, activity of creatine kinase and nucleoside-diphosphate kinase while adenylate kinase was absent [21, 22].

Electron microscopic localisation of mitochondrial creatine kinase

These results suggested that contacts may reflect the functional interaction of components in the outer mitochondrial compartment (schematically shown in Fig. 3). Based on kinetic analyses of surface bound kinases such as glycerol kinase [5–7] and hexokinase [3, 4, 6], surface proteolysis analysis [22, 23], electron microscopy [20, 21], and interaction with specific antibodies [22], a structural organisation of the functional complexes was postulated as depicted in Fig. 3. It was assumed that the peripheral kinases might interact with the adenylate translocator via the pore protein, while kinases in the intermembrane space such as creatine kinase might functionally couple porin and outer membrane with the adenylate translocator and inner membrane [13, 24]. The capacity of the octamer of creatine kinase to connect two artificial membranes supported this idea [25].

In order to localise mitochondrial creatine kinase by specific antibodies, it was necessary to overcome the outer membrane barrier. This was performed either by reaction of the antibodies with sections of Lowicryl embedded samples of chicken retina (post-embedding) or by pre-treatment of isolated rat brain mitochondria with digitonin (pre-embedding). It was known from previous investigations that about 60% of the mitochondrial creatine kinase as well as hexokinase remained fixed to the inner membrane matrix fraction after incubation with 100–300 µg digitonin per mg of mitochondrial protein [13].

The application of the two different techniques resulted in the observation of two fractions of mitochondrial creatine kinase. In sections of retina, decorated post embedding (Fig. 5), the enzyme was located at the periphery between the two envelope membranes and along the

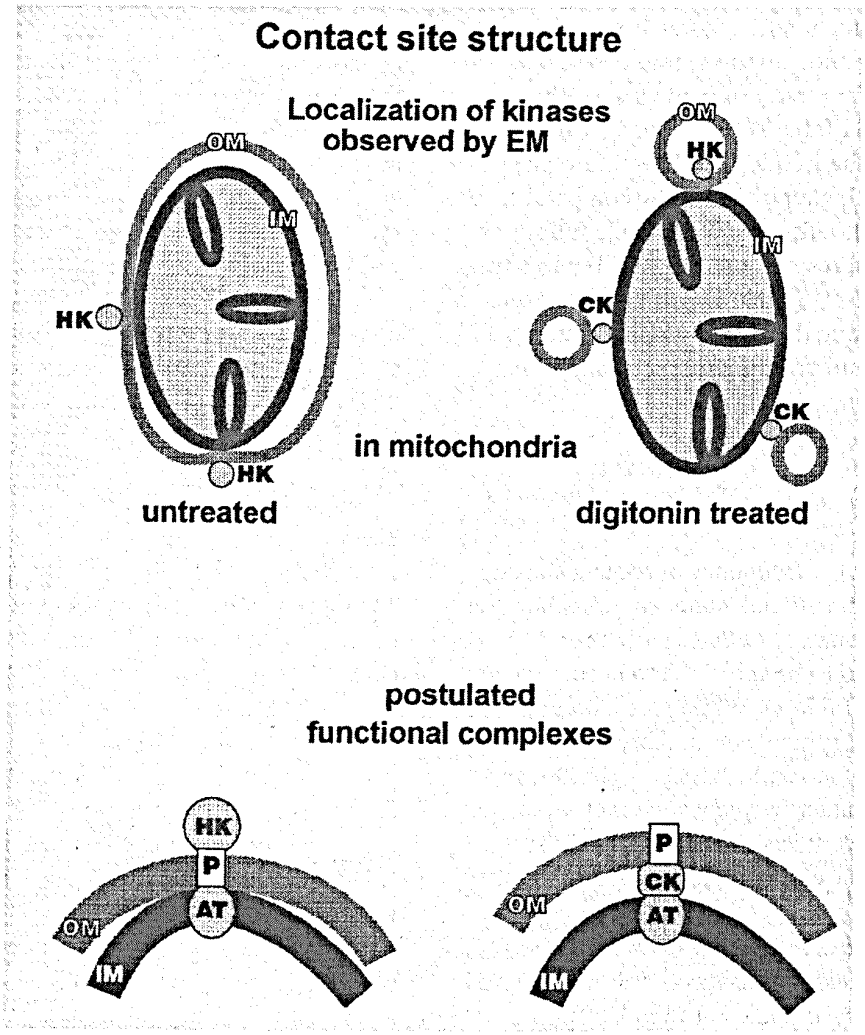


Fig. 3. The upper part of the figure schematically depicts the localisation of hexokinase in intact liver and brain mitochondria as observed by electron microscopy in Ref. 20 and 21 and the localisation of hexokinase in liver- and creatine kinase in brain mitochondria after digitonin treatment (shown in Fig. 4A–C and D). The lower part of the figure presents the postulated functional complexes derived from the electron microscopic and kinetic analyses. HK = hexokinase, CK = creatine kinase, AT = adenylate translocator, P = porin, OM = outer membrane, IM = inner membrane.

mitochondrial cristae, while pre-embedding staining of digitonin treated mitochondria led to an exclusive peripheral immuno reactivity (Fig. 4D). Closer analysis [58] of the distribution of the peripheral creatine kinase resulted in the observation that 43% was found in the contact points where after digitonin treatment remnants of the outer membrane remained attached (Fig. 4D large arrowheads). Since small fragments of the outer membrane, not capable of forming vesicles (Fig. 4D small arrowheads), may not have been identified as contacts, this relatively high amount of gold grains in identified attachment points means that presumably most of the peripheral mitochondrial creatine was organised in the contact sites.

Despite the exclusive peripheral localisation of mitochondrial creatine kinase found by Biermans *et al.* [26] and by contact site isolation [13, 21, 22], we concluded on the basis of our results with post embedding staining (Fig. 5), and in agreement with Wegman *et al.* [27] and Hemmer *et al.* [28], that the enzyme has a second intramitochondrial location along the surface of the crista membranes in brain mitochondria. The mainly peripheral localisation of mitochondrial creatine kinase in the case of pre-embedding labelling can easily be explained by the fact that the enzyme in the intracristae space was not accessible to antibodies although the mitochondria were treated with digitonin (Fig. 4D). As described earlier, we were able to separate from disrupted brain mitochondria a contact site fraction (containing peripheral creatine kinase) and an inner memrane-matrix fraction

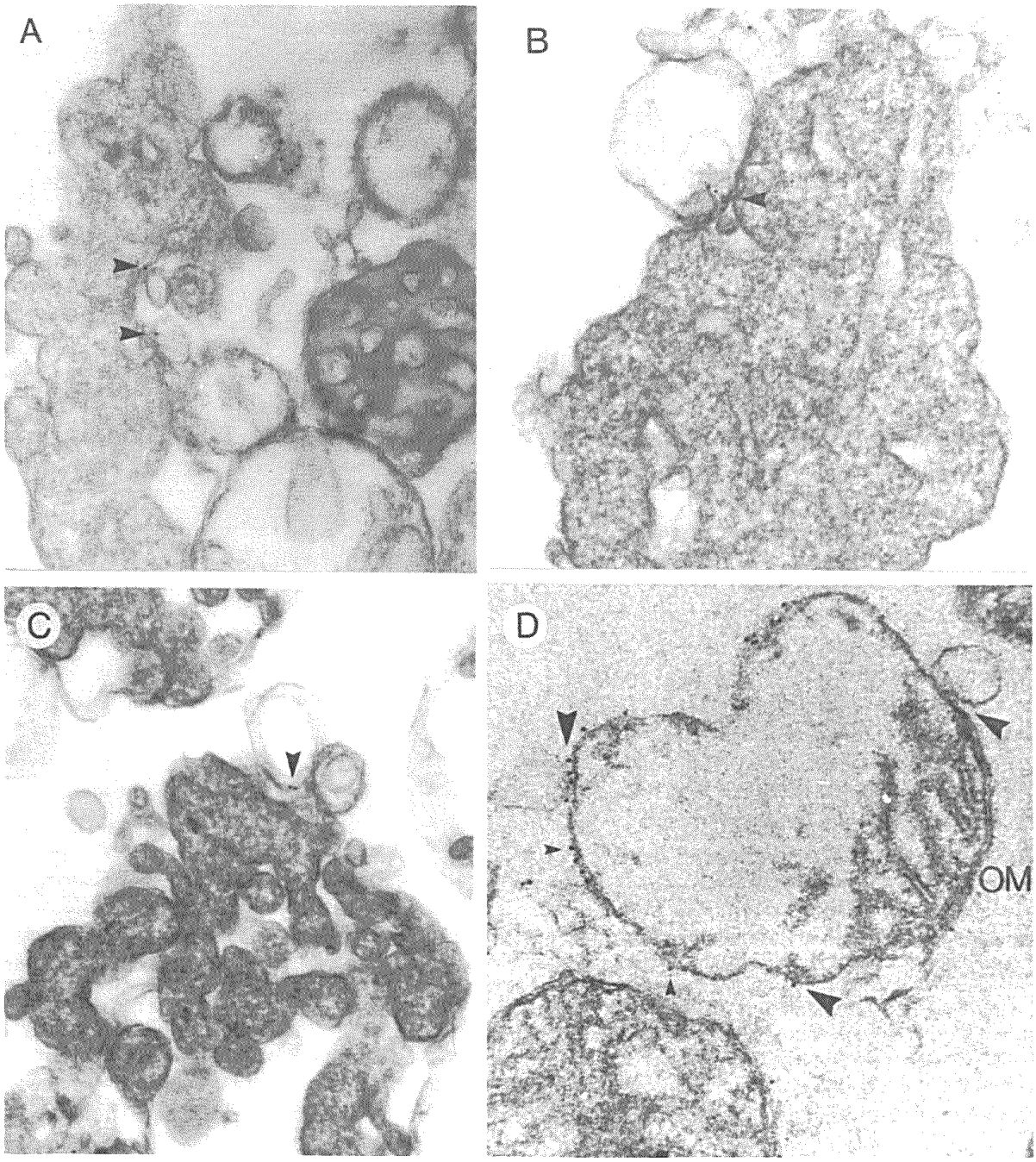
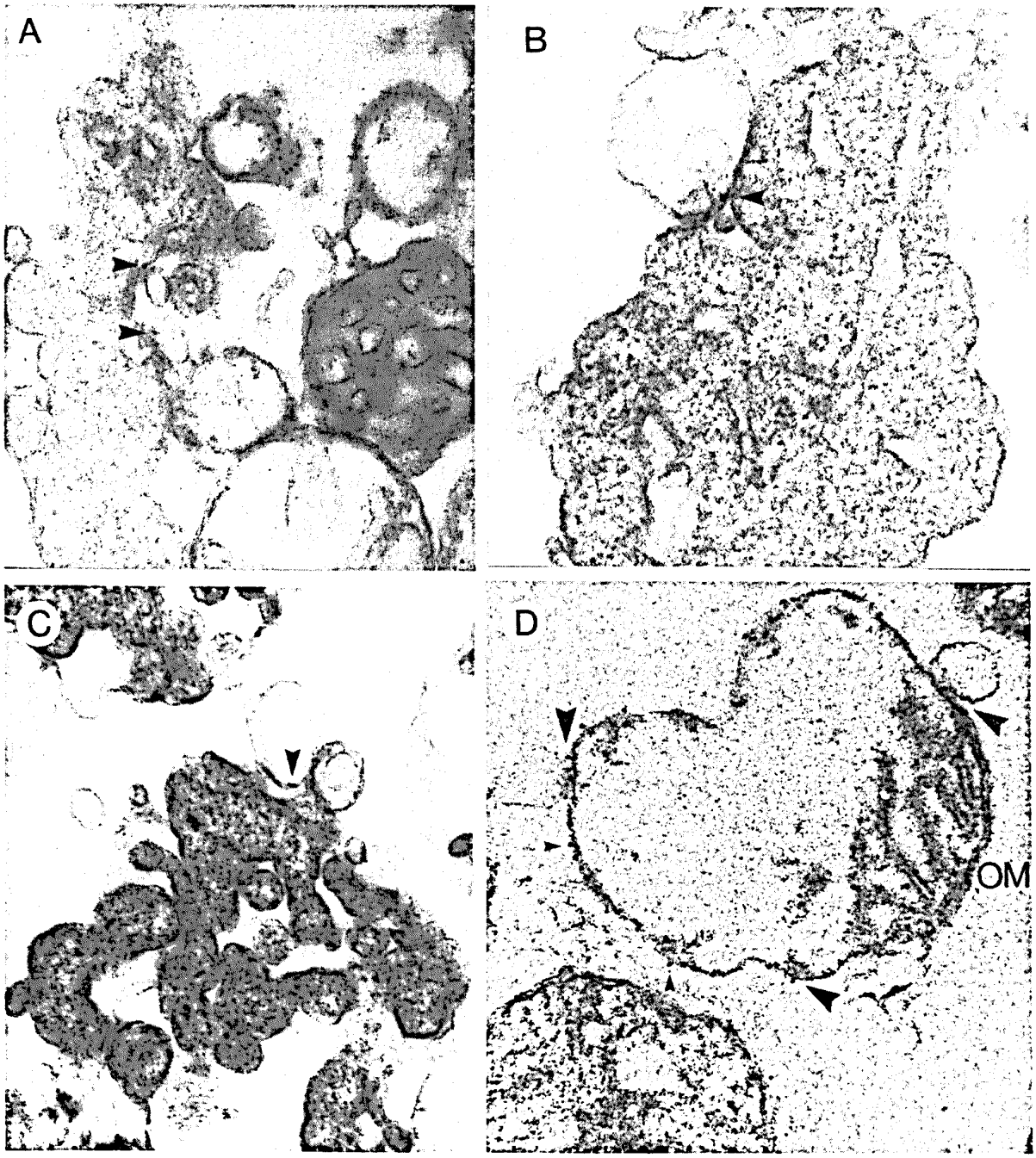


Fig. 4. Preembedding labelling of hexokinase and mitochondrial creatine kinase in isolated, digitonin-treated liver and brain mitochondria. A, B, C): Intact liver mitochondria labelled with hexokinase antibodies and protein A-gold and subsequently treated with 100 µg digitonin/mg of mitochondrial protein. The gold grains left at the surface of the inner membrane were exclusively found in vesicles of the outer membrane which remained bound to the inner envelope at the contact sites (arrowheads). No label was attached to the free surface of the inner membrane. Magnification 70,000. D): Brain mitochondria treated with 200 µg/mg digitonin and subsequently incubated with antibodies against mitochondrial creatine kinase and protein A-gold. Parts of the outer membrane remained attached to the inner membrane either forming vesicles (large arrowheads) or small patches (small arrowheads). It is striking that creatine kinase is often located (large arrowheads) between outer membrane vesicles and the inner membrane. In general only parts of the free accessible inner membrane are labelled whereas the outer membrane (OM) is free of gold grains. Magnification 60,000. *methods:* Mitochondria from rat liver were incubated intact for 10 min at room temperature with polyclonal antiserum against brain hexokinase, whereas brain mitochondria were treated with 200 µg digitonin per mitochondrial protein and than incubated with antibodies against brain mitochondrial creatine kinase. The antibodies were diluted in blocking solution (0.2% gelatine and 0.5% bovine serum albumin in sucrose-Hepes medium). Samples were sedimented by 45 sec centrifugation in a tabletop centrifuge followed by a washing step in sucrose-Hepes medium. Mitochondria were then incubated for 10 min at room temperature with protein A-gold diluted in blocking solution and were again centrifuged and washed in sucrose-Hepes medium. Liver mitochondria were subsequently treated with 100 µg digitonin per mg. The mitochondrial sediments of liver and brain were fixed for 45 min on ice with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, washed several times in 0.1 M cacodylate, 0.15 M sucrose, and post fixed for 2 hours with 1% OsO_4 in 0.1 M cacodylate, 0.11 M sucrose. After washing in 0.1 M cacodylate with 0.15 M sucrose, the samples were subjected to stepwise dehydration with acetone and embedded in Spurr's low viscosity resin.



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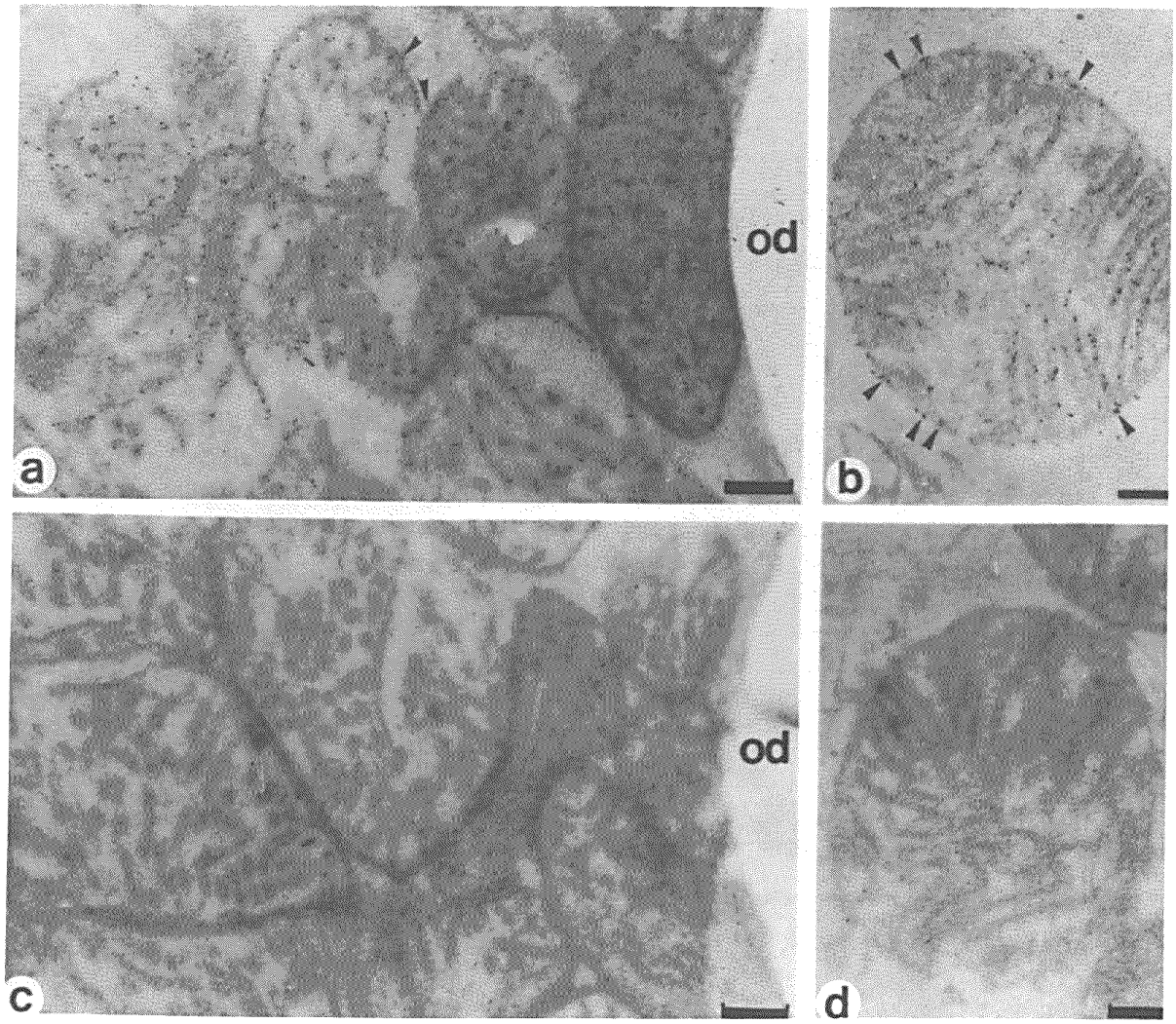

Fig. 5. Post-embedding immuno-gold labelling of mitochondrial creatine kinase in chicken retina photoreceptor cells. a) shows at lower magnification parts of the inner segment at the level of the ellipsoid portion, where mitochondria are clustered. b) single mitochondrion in the same region at higher magnification. Note the dual localisation of mitochondrial creatine kinase along the entire cristae membranes as well as at the periphery, where inner and outer membrane are in close proximity (see arrow-heads in a) and b)). c) and d) depict control sections of the same tissue region as above stained with pre-immune serum and the same second antibody as used in a) and b). There is no significant immuno-gold labelling. od = oil droplets of chicken photo receptor cells. Note the dual localisation of mitochondrial creatine kinase at the periphery and along the cristae. Bar = 250 nm. *Methods:* Chicken eye cups were prefixed with 3% paraformaldehyde/0.1% glutaraldehyde in PBS for 30 min at 0° C. Retinas were removed and fixed for an additional 2 h in the same fixative. Dehydration and embedding in Lowicryl K4M were carried out according to [52] in a low-temperature embedding apparatus at – 35° C. Infiltration with liquid Lowicryl K4M resin was performed at – 35° C as described in [53]. 70 nm sections were cut and immuno labelled according to [27]. An affinity-purified rabbit anti-chicken antibody against mitochondrial creatine kinase was used followed by goat anti-rabbit IgG coupled to 5 nm colloidal gold particles. Sections were stained with 2% aqueous uranyl acetate for 5 min followed by lead citrate for 1 min and examined in a JEOL 100 electron microscope.

(containing central creatine kinase). However we determined creatine kinase activity almost exclusively in the contact site fraction, suggesting that, like the antibodies, also access of substrates to the enzyme in the inter-cristae space was restricted [21, 22].

In view of these differences in accessibility between the peripheral and central creatine kinase activity one might assign a specific structural and regulatory function to the peripheral mitochondrial creatine kinase in situations of short and rapid energy requirements, while the activity of mitochondrial creatine kinase located along the cristae membranes may be recruited in the case of prolonged and large energy requirement. In addition, it is tempting to speculate that the relative proportion of cristae versus contact site creatine kinase may

change in a dynamic way depending on the actual requirements in energy flux [26, 29].

Effect of macromolecules on the binding of hexokinase

It was observed that hexokinase had a higher affinity to isolated contact sites than to outer membrane [23, 30] which agreed with the electron microscopic localisation of hexokinase in zones of boundary membrane contacts, and the correlation of bound hexokinase activity with the frequency of contacts summarised in Table 1. In general these findings indicated on one hand a specific regulatory role of the contact sites in complex formation with hexokinase and on the other hand suggested that hexokinase binding could be used to look into changes in contact site frequency. We, therefore, used hexokinase binding to follow the effect of macromolecules on the reconstitution of contact sites and the structure of the outer mitochondrial compartment, respectively [14].

It was found that the half maximal saturation by hexokinase of the surface of liver mitochondria was significantly lower in the presence of 10% dextran with a Mr of 70 kDa than compared to the control. In general the binding of hexokinase to contact sites appeared to be a co-operative process and led to a 5 to 10 fold activation [14, 20, 31] of the enzyme. When, however, contacts were suppressed by addition of 20% glycerol or 50 μM dinitrophenol we observed almost the same binding as to isolated outer membrane, which was characterised by a hyperbolic saturation curve and no activation of the enzyme. The results indicated two aspects: i) that additional contacts may have been restored in the presence of dextran and ii) that the function of contacts in case of hexokinase was to increase the binding affinity to the membrane surface and to activate the enzyme.

Structure of the hexokinase porin complex

Supposing that hexokinase bound to the pore protein in the contact sites, the cooperativity of the binding in the contact sites and the activation of the enzyme suggested the formation of oligomers in the complex. In support of this assumption Xie and Wilson, by cross-linking experiments, observed that hexokinase I formed tetramers upon binding to mitochondrial membranes [32]. We therefore screened for conditions of hexokinase porin interactions which caused activation of the enzyme.

Hexokinase I was isolated according to Wilson [33] and incubated with porin prepared from liver mitochondria in lauryl dimethyl amino oxide (LDAO) as described by DePinto [34]. The enzyme was activated 5–10 fold under conditions such as learned from binding of hexokinase to intact mitochondria: pH-(6.5), dependence on Mg^{2+} (10 mM). The activation of the enzyme by porin was inhibited by glucose-6-P (5 mM). Hexokinase was not activated (or did not interact?) with porin prepared in Triton X-100. This suggested that the porin N-terminus must be available for interaction with hexokinase, which agreed with the fact that antibodies against the porin N-terminus suppressed the activation of hexokinase by porin in LDAO (Wicker *et al.* unpublished). The N-terminus was found to be susceptible to proteases in porin prepared with LDAO in contrast to preparations of porin in Triton X-100 [35]. In intact mitochondria the porin N-terminus was exposed at the outer surface of mitochondria [35]. Analysis of the hexokinase/porin mixture by column chromatography [36] revealed activity of porin and hexokinase in a protein fraction of 480 kDa, suggesting the formation of a complex composed of a hexokinase tetramer and presumably porin dimer. A dimer of free hexokinase (170 kDa) and hexokinase fragments (76 kDa) as well as porin (30 kDa) were additionally eluted from the column. As indicated by SDS-gel electrophoresis and immuno blots with antibodies against hexokinase I and the N-terminal end of porin [these were a gift of Dr. Thinnes, Göttingen] porin and hexokinase were present in the 480 kDa fraction from the column. We were able to reconstitute the pore in the complex with hexokinase in artificial membranes, and to measure the voltage dependence of the conductance [36]. It was observed that the pore was less voltage sensitive, suggesting that the interaction with hexokinase might physiologically allow better permeation of adenine nucleotides (see below).

Effect of macromolecules on the attachment of mitochondrial surface components

The observation of a higher hexokinase affinity to contact sites suggested that more hexokinase might remain bound when the structure of the outer compartment would be preserved by the presence of dextran during isolation. We thus isolated liver mitochondria in a medium containing 140 mM KCl, 10 mM Hepes pH 7.4 and 15% dextran 20 and for comparison in 250 mM sucrose/10 mM Hepes. The specific activity of hexokinase in the mitochondrial fraction was 2.5 times higher compared to the control mitochondria (Table 2). Supposing that contacts were responsible for binding of hexokinase with higher affinity, these results suggested that isolation of mitochondria in the presence of macromolecules

might preserve contacts sites, and thus structures of the outer mitochondrial compartment. In general these results show that substitution of macromolecules during mitochondrial isolation not only preserves the outer mitochondrial compartment but in addition may have important consequences for the attachment of all kinds of regulatory proteins to the mitochondrial surface.

Function of the hexokinase porin complex in energy metabolism

The induction of contact sites by dextran and the influence on activation of hexokinase rose the question of the function of the hexokinase porin complexes. Besides of the activation of the enzyme we assumed a function in regulation of energy metabolism. Bessman and co-workers have produced evidence that the mitochondrial hexokinase [3, 4] preferentially utilises intramitochondrially generated ATP. They have also shown that this preference decreased with increasing total ATP concentrations. The same was observed in reticulocyte [37] and brain [38, 39] mitochondria. These experiments were performed in the absence of dextran. Addition of dextran to brain mitochondria, however, resulted in changes of the kinetic properties of the originally bound enzyme, which pointed to a functional coupling with the AdN-translocator [14, Fig. 6]. In agreement with Bessman and co-workers [3, 4] we noted a significantly lower Km for ADP (ATP) when the enzyme was utilising internal ATP as compared to external ATP (Fig. 6B). As observed by others [38, 39], the Vmax with internal ATP was about half of that with external ATP (compare Fig. 6A with 6B). The addition of dextran had no significant effect on these parameters, suggesting that contact site formation did not improve the supply of hexokinase with internal ATP [14, Fig. 6B]. On the other hand, when we studied the stimulation of the oxidative phosphorylation by the ADP formed via the activity of originally bound hexokinase, we noticed a significant decrease in Km and a duplication of the catalytic efficiency (Vmax/Km) in the presence of dextran (Fig. 6C). The Vmax of hexokinase in this experiment (calculated from the O2 consumption and the ADP/O ratio) was almost that determined with external ATP (Fig. 6A) but the Km was decreased by dextran to the values observed with internal ATP (Fig. 6B). Thus the induction of contacts by dextran appeared to improve the saturation of the adenine nucleotide translocator with ADP (in the presence of increasing concentrations of external ATP) because of facilitated communication with hexokinase. On the whole the experiments emphasise the importance of contacts in the stimulation of the oxidative phosphorylation via peripheral kinases.

Based on their results, Bessman and co-workers [3, 4, 40], about 25 years ago, postulated a direct coupling between the adenylate translocator and hexokinase. It was subsequently shown that hexokinase specifically binds to the outer membrane pore [1, 2]. However, direct evidence of coupling between porin and the adenylate translocator has not yet been provided. We found that the relation of the translocator to the crista membrane marker cytochrome oxidase was 3 times higher in the isolated contact site fraction from liver mitochondria compared to the corresponding inner membrane, this was, however, not observed in subfractions of kidney mitochondria [41]. Evidence for a direct interaction between the translocator and porin came recently from the characterisation of the isolated mitochondrial benzodiazepine receptor from kidney mitochondria [42]. In these

Table 2. Effect of dextran addition during isolation of rat liver mitochondria on the activity of mitochondrial bound hexokinase

	HK mU/mg		G-6-Pase mU/mg		SDH U/mg		MAO mU/mg	
	Homog	Mitoch	Homog	Mitoch	Homog	Mitoch	Homog	Mitoch
Dextran	4.09	9.83	1.02	0.07	0.09	0.24	1.14	3.33
	± 2.07	± 5.01	± 0.39	± 0.03	± 0.05	± 0.13	± 0.55	± 1.21
Control	3.38	3.83	1.24	0.08	0.12	0.31	0.90	3.74
	± 1.65	± 1.65	± 0.06	± 0.01	± 0.07	± 0.12	± 0.34	± 1.66

The specific activity of hexokinase is compared to that of marker enzymes for endoplasmic reticulum ER (G-6-Pase), inner- (SDH) and outer mitochondrial membrane (MAO) in the homogenate (= Homog) and mitochondrial fraction (= Mitoch). While the specific activity increase of marker enzymes of inner and outer membrane in the mitochondrial fraction was comparable (2–2.5 fold) in control and dextran containing medium, the specific hexokinase activity in the mitochondrial fraction was 2.5 times higher in the presence of dextran compared to the control. As judged from the G-P-ase activity, the contamination by ER was comparable in both media. *Methods:* Mitochondria were isolated from a Teflon potter homogenate by fractional centrifugation in either 0.25 M sucrose, 10 mM Hepes pH 7.4 (= Control) or 140 mM KCl, 15% dextran 20 and 10 mM Hepes pH 7.4 (= Dextran). Mean of 8 experiments.

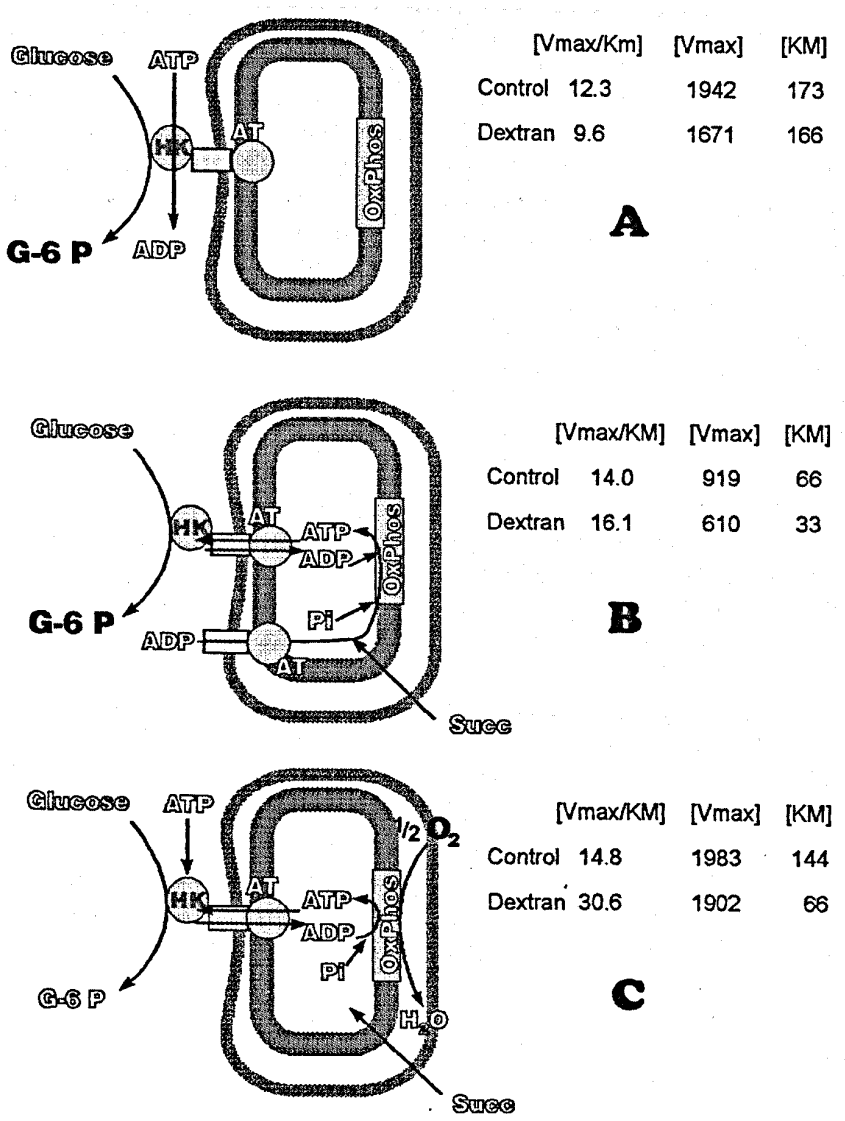

Fig. 6. Kinetic analysis of bound hexokinase in isolated mitochondria from rat brain. The experiments show the kinetic advantage of a functional coupling between hexokinase and adenylate translocator in the contact sites. When contacts were induced by dextran in experiment C, the velocity of hexokinase was as high as in A (with external ATP) while the Km was comparable to that observed in B with ATP produced by oxidative phosphorylation. *Methods:* The activity of originally bound hexokinase was analysed in 0.25 M sucrose/10 mM Hepes medium in the presence 2 mM glucose and 10% dextran 70 as indicated. The velocity of the activity was determined by registration of glucose-6-phosphate (G-6 P) formation by optical test [54] in experiment A and B, while the stimulation of state 3 respiration ($1/2O_2$) was measured by oxygen electrode in experiment C. In experiment B ATP was formed via activity of oxidative phosphorylation in the presence of 5 mM succinate (Succ) and 5 mM phosphate (Pi) and 2 mM di-adenosine 5'-pentaphosphate. In experiment C the ADP to stimulate the oxidative phosphorylation was formed by the activity of hexokinase in the presence of 5 mM succinate and 5 mM phosphate. Hexokinase activity was calculated from the ADP/O ratio and the oxidation rate per min and ml mitochondrial suspension. The kinetic data shown in the figure were taken from [14] and are mean of 3 experiments.

experiments a protein complex of Mr 72 kDa was purified. On SDS-PAGE the mitochondrial benzodiazepine receptor appeared to be composed of three polypeptides which were identified as porin and adenylate translocator and a protein of Mr 18 kDa with so far unknown function which bound the benzodiazepine and was purified and sequenced by Krueger *et al.* [43].

Direct effects of macromolecules on the outer membrane pore

The compartmentation of adenine nucleotides in the mitochondrial inter-membrane space has been observed by several authors but the reason for this was still unclear. From radioisotopic measurements Viitanen *et al.*

concluded that the outer membrane represents a diffusion barrier for creatine kinase since compartmentation effects were reduced by removing the outer membrane [44], whereas we found in digitonin treated mitochondria that the enzyme was still not free accessible to negatively charged substances [13]. Brooks *et al.* came to the same conclusion [45]. These findings pointed to a somehow restricted AdN exchange through the outer membrane pore. The pore protein in the outer membrane is known to be voltage dependent [46]. The conductance of the pore, reconstituted in artificial membranes, decreases to about 50% at a voltage above 30 mV. The pore in this low conductance state was found to be cation selective [47]. It was assumed that the inner membrane potential might affect the pore in the contact sites, where a capacitive coupling between the two envelope membranes appeared possible [47]. However, Zimmerberg and Parsegian [48] observed that the presence of macromolecules increased the voltage sensitivity of the reconstituted pore. In agreement with these results, we demonstrated recently that addition of 10% of different dextrans of Mr between 20 kDa and 500 kDa led to pores in the low conductance state already at 10 mV transmembrane potential [15]. In view of these results, we assume that physiologically, besides the effect of cytosolic proteins on the structural organisation of the outer compartment, also a direct effect might occur on the permeability of AdN through all outer membrane pores.

Effects of macromolecules on the exchange of adenine nucleotides in isolated mitochondria

The functional consequences of this limitation were recently demonstrated by the observation that addition of 10% dextran to conventionally isolated liver mitochondria affected the export of ADP as well as the import into the inter-membrane space as is described in [15]. The ADP formed in the inter-membrane space by adenylate kinase was not readily accessible to extramitochondrial pyruvate kinase, and vice versa the adenylate kinase originally located in the inter-membrane space was less accessible to external ADP. The reduction of ADP diffusion correlated with the concentration of added dextrans of different Mr [15].

Conclusions

The important role of the outer mitochondrial compartment in regulation of the mitochondrial energy metabolism is based on the following aspects: i) the outer membrane pore is physiologically not freely permeable for adenine nucleotides so that the space between the two envelope membranes must be regarded as a separate compartment, ii) the outer compartment contains a number of different molecules located at the surface of the membranes, integrated into the membranes and between the membranes which form transient, functional complexes in the presence of macromolecules. Examples of the dynamics of these complexes are the specific binding of hexokinase to the contact sites where the enzyme associates as a tetramer [36] and the specific formation of creatine kinase octamers, consisting of 4 dimers as active building units, in these sites [13]. The functional advantage of these complexes presumably composed of the kinases, porin, and adenylate translocator would be molecular channelling of ADP into the inner compartment in order to keep a high phosphorylation potential in the cytosol also while ATP turnover is high. In this way, the mitochondrial energy metabolism can be activated (without general ADP increase) during rapid ATP turnover in muscle and brain by an interplay between cytosolic and mitochondrial creatine kinases supplying the intramitochondrial ADP and by bound hexokinase providing mitochondrial substrates via glucose phosphorylation (Fig. 7). On the other hand, also at rest, the activation of the mitochondrial metabolism via the kinases would be advantageous. Because ion pumps such as Ca^{2+} ATPase in the endoplasmic or sarcoplasmic reticulum depend very critical on the available free energy [49], large fluctuations of the phosphorylation potential must be avoided. Thus, if increased blood glucose levels, followed by high activity of glycogen synthesis in liver and resting muscle, demand activation of oxidative phosphorylation, this may be performed via bound hexokinase and nucleoside-diphosphate kinase (Fig. 7) which channel the ADP into the inner compartment avoiding aequilibration with the cytosolic AdN levels.

Correlated action of Ca^{2+} and ADP supply in regulation of energy metabolism

It has been proposed that Ca^{2+} ions serve as a signal to regulate oxidative phosphorylation on one hand by direct activation of mitochondrial ATP synthase [55] and on the other hand by activation of substrate supplying enzyme reactions (pyruvate-dehydrogenase, NAD-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase [56, 57]). This does not lower the importance of ADP supply by kinases discussed above in regulation of

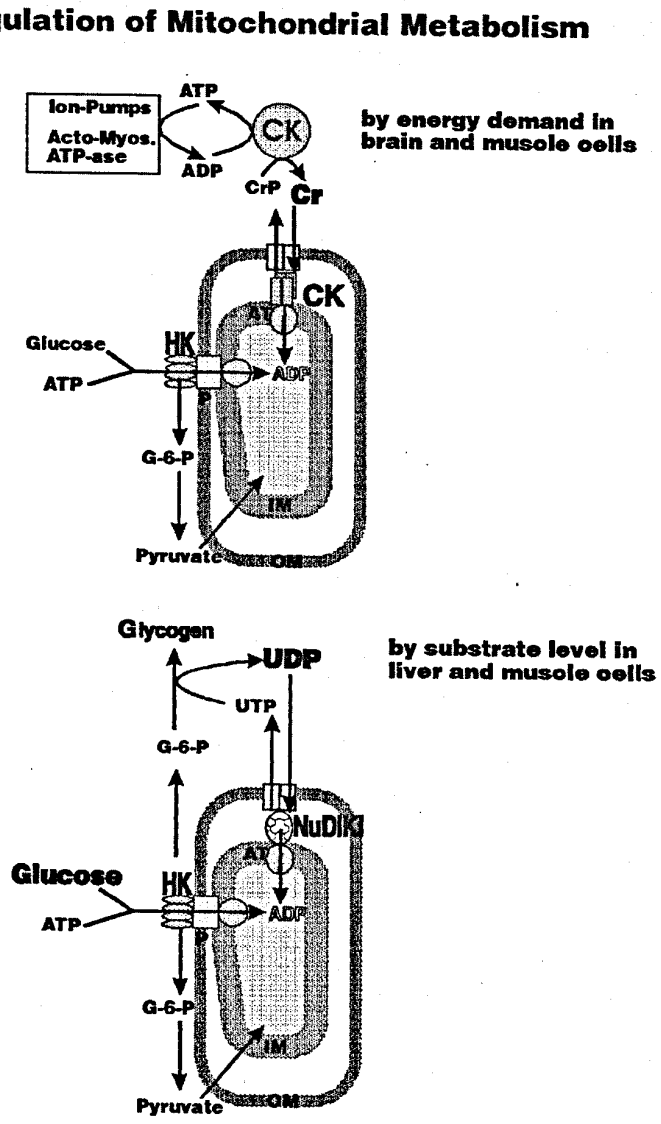

Fig. 7. Schematic representation of different ways to regulate the oxidative phosphorylation without changing cytosolic ATP and ADP levels. The intramitochondrial ADP is indirectly increased by the activity of peripheral kinases. A) in case of rapid ATP turnover upon stimulation of muscle or nerve cell activity, the level of creatine (Cr) increases and activates mitochondrial creatine kinase (CK) to produce intramitochondrial ADP. In parallel glucose uptake is increased by activation of hexokinase (HK). B) in case of liver cells or muscle cells at rest, an increased level of glucose and of the UDP, produced during glycogen synthesis, rises intramitochondrial ADP via bound hexokinase (HK) and nucleoside-diphosphate kinase (NuDiKi). In the contact sites, hexokinase and mitochondrial creatine kinase (which is a dimer in the active state) form tetramers and octamers, respectively.

mitochondrial activity but in contrast emphasises the function of the ADP producing reactions as a prerequisite of Ca^{2+} dependent activation. In view of this correlated action, it is worthwhile to mention that a Ca^{2+} binding protein was found to be concentrated in the contact sites of brain mitochondria [21] suggesting that Ca^{2+} might induce the formation ADP producing kinase complexes.

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