The intra-mitochondrial cytochrome c distribution varies correlated to the formation of a complex between VDAC and the adenine nucleotide translocase: this affects Bax-dependent cytochrome c release

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Abstract

The mechanism of Bax-dependent cytochrome c release is still controversial and may also depend on the actual localisation of cytochrome c. (i) we studied the distribution of cytochrome c in sub-fractions of rat kidney mitochondria and found that 10–20\% of the total cytochrome c was associated at the peripheral inner membrane and to some extent organised in the contact sites. (ii) Cytochrome c concentrations in the contact site fractions varied related to surface bound hexokinase activity. It decreased upon reduction of contact sites by glycerol or specific dissociation of the VDAC–ANT complexes by bongkrekate, whereas it increased upon induction of contacts by dextran or association of VDAC–ANT complexes by atracyloside. (iii) The outer membrane pore (VDAC) acquires high capacity for hexokinase binding by interacting with the ANT. Thus, surface-attached hexokinase protein indicated the frequency of VDAC–ANT complexes and the correlation between hexokinase activity and cytochrome c suggested association of the latter to the complexes. (iv) Substances affecting exclusively the structure of either hexokinase (glucose-6P) or cytochrome c (borate) led to a decrease only of the effected protein without changing the concentration of other contact site constituents. (v) Hexokinase was furthermore used as a tool to isolate the contact site forming complex of outer membrane VDAC and inner membrane ANT from Triton-dissolved membranes. Cytochrome c remained attached to the hexokinase VDAC–ANT complexes that were reconstituted in phospholipid vesicles. (vi) The vesicles were loaded with malate and Bax\textsuperscript{D}C released the endogenous cytochrome c from the reconstituted complexes without forming unspecific pores for malate. Bax\textsuperscript{D}C targeted a cytochrome c fraction associated at the VDAC–ANT complex. The cytochrome c organisation was dependent on the actual structure of VDAC and ANT. Thus, the Bax\textsuperscript{D}C effect was suppressed either by hexokinase utilising glucose and ATP or by bongkrekic acid both influencing the pore and ANT structure.

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1. Introduction

There are several signal- and tissue-specific pathways to induce apoptosis. One signal is the release of cytochrome c from mitochondria [1–3] that activates caspases [2] by binding to a cytoplasmic protein Apaf-1 in presence of dATP [4]. An important stimulus which causes cytochrome c release is the pro-apoptotic Bcl-2 family member Bax (for review see Ref. [5]). It is synthesised in the cytoplasm and translocated to mitochondria [6] where it releases cytochrome c [7,8].

The mechanism by which Bax releases cytochrome c from the mitochondrial inter-membrane space into the cytoplasm is still controversial. Some results indicate that Bax induces permeability transition (PT), leading to swelling of mitochondria, followed by rupture of the outer membrane and release of several factors into the cytoplasm [9]. In many cases of apoptosis, mitochondrial depolarisation, swelling and release of larger proteins occurs as a late
event, well after the release of cytochrome c [9,10]. In these cases, Bax-induced cytochrome c release appears to be more selective: (i) it amounts to approximately 20% of the total cytochrome c, (ii) it is not dependent on outer membrane disruption and PT.

Full-length Bax itself formed pores for small molecules in synthetic bilayers [11,12] and after reconstitution in liposomes [13]. In contrast, BaxΔC did not form pores permeable for cytochrome c in outer membranes free of contact sites or vesicles with reconstituted VDAC [14], but was well able to release cytochrome c from intact mitochondria [7,8].

So far, most investigations have not thought of the cytochrome c compartmentation and a possible different organisation in intact mitochondria compared to lipid vesicles. Considering the new mitochondrial structure model [15,16], we suggest that different fractions of cytochrome c associated to different membrane areas of the inner membrane have to be regarded such as cristae membranes, inner boundary membrane and contact sites in addition. Energy transferring contact sites are formed by interaction between the outer membrane pore (VDAC) and the ANT [17–19]. VDAC, being in a complex with ANT, exerts higher capacity for hexokinase binding [20] compared to VDAC beyond the contact sites. Thus, hexokinase could be used as an indicator of VDAC–ANT complexes and a marker enzyme of contact sites [21,22]. The complex between hexokinase, VDAC and ANT is relatively stable as it remains intact in a Triton extract of mitochondrial membranes from rat brain [23]. This observation led us to use the enzyme as an instrument to separate by anion exchange chromatography the hexokinase contact sites from other contact site-forming complexes such as the VDAC creatine kinase ANT complex. When the different contact sites were reconstituted into phospholipid vesicles, hexokinase as well as creatine kinase was functionally coupled to the ANT, phosphorylating external glucose or creatine by utilising internal ATP [23]. This process could be blocked by ANT inhibitors bongkrekate or atraclytamide.

It will be shown that a significant amount (20% of total) of cytochrome c is associated with the hexokinase-containing contact sites. It was therefore tempting to study the effect of Bax exclusively on this cytochrome c fraction by applying the known technique of isolation and reconstitution of the hexokinase VDAC–ANT complexes [23,24]. This was particularly interesting as recent investigations showed that Bax and hexokinase compete for the same binding sites in hepatocyte mitochondria [25]. Furthermore, protein kinase B linked suppression of cytochrome c release and apoptosis was found to depend on the activity of mitochondrial bound hexokinase [26,27]. In general, we assumed that this contact site fraction could be the mitochondrial cytochrome c pool specifically releasable in the early period of Bax action [28].

2. Material and methods

2.1. Materials

All reagents were purchased from Sigma or Roche if not stated otherwise.

2.1.1. Isolation of kidney mitochondria

The three kidneys from 3–4-month-old male Wistar rat were homogenised in 30 ml HEPES-sucrose buffer containing 250 mM sucrose, 10 mM HEPES NaOH pH 7.4, 0.5% BSA (essential fat-free), 1 mM reduced glutathione (GSH), 1 mM EGTA using a glass Teflon potter homogeniser. The homogenate was centrifuged for 10 min at 700 × g. The supernatant was centrifuged for 10 min at 7000 × g. The pellet was re-suspended in 15 ml sucrose-dextran buffer containing the same medium as above but 10% dextran 15,000 in addition. The suspension was centrifuged for 12 min at 700 × g. The supernatant was centrifuged for 20 min at 7000 × g. Mitochondria were re-suspended in 200 μl KCl-Dextran buffer containing 125 mM KCl, 10 mM HEPES pH 7.4, 1 mM GSH.

2.1.2. Isolation of contact site fraction from kidney mitochondria

Contact sites were isolated by sucrose density gradient centrifugation from osmotically shocked kidney mitochondria according to the method described by Adams et al. [21]. The method was slightly modified by omitting the sonification step after osmotic shock and creatine kinase activity was measured in the presence of 0.2% Triton X-100.

2.1.3. Isolation of hexokinase porin–ANT complexes from brain membranes

The method was performed essentially as described recently [23,24] with slight modifications. Rat brain, stored frozen, was homogenised by a Teflon potter in isolation medium (10 mM Heps, pH 7.4, 100 mM glucose, 1 mM monothioglycerol) at approximately 10 ml per gram of wet tissue. The homogenate was centrifuged 10 min at 4 °C and 10,000 × g in a “Sorvall” centrifuge with rotor SS-34. The pellet was re-suspended in the same volume of isolation medium and the centrifugation was repeated. This process removed only 10% of total hexokinase from rat brain sediment. The last pellet was re-suspended in the isolation medium containing 1% Triton X-100 and was incubated for 45 min at room temperature with gentle stirring. The insoluble membrane material was removed by centrifugation at 45,000 × g in a Beckman L8-M ultracentrifuge, using rotor Ti-50, for 45 min at 4 °C. The supernatant was incubated with DE 52 cellulose (Whatman) that had been equilibrated with 5 mM Tris-phosphate, pH 8.0, 100 mM glucose, 1 mM dithioerythritol. The DE 52 cellulose was loaded with the extract and packed as a column, from which hexokinase activity and
cytochrome c was eluted by a KC1 gradient between 100 and 400 mM and determined in the fractions by optical test as described below.

2.1.4. Reconstitution of hexokinase porin–ANT complexes

Lipid mixture used in reconstitution was phosphatidylcholin containing 2% cholesterol. The phospholipid vesicles, 0.3% n-octyl-β-D-glucoside and 5 ml of the combined hexokinase peak fractions from the DE 52 were mixed for 20 min at room temperature, followed by overnight dialysis at 4 °C against 125 mM sucrose and 10 mM Hepes, pH 7.4. The vesicles were loaded with 10 mM KC1 and 5 mM malate by sonification followed by subsequent chromatography on Sephadex G 50 using a 24/1 cm column with a flow rate of 20 ml per hour. The column was equilibrated in 125 mM sucrose and 10 mM Hepes pH 7.4. After chromatography, the vesicle permeability for malate was tested. This was performed by centrifugation of the vesicles for 45 min at 400,000 × g followed by malate determination in the supernatant and sediment.

2.1.5. Enzyme assays

Hexokinase (EC 2.7.1.1) activity was measured in agreement with Bücher et al. [29]. The enzyme was determined in a coupled optical enzyme assay with glucose and ATP as substrates. Bacterial glucose 6-phosphate dehydrogenase and NADH formation were used as indicators.

The determination of succinate dehydrogenase (SDH) (EC 1.3.9.9) activity was performed as described in Brdiczka et al. [30].

Malate was determined by an enzyme assay according to Bergmeyer [31].

2.1.6. Cytochrome c determination

Cytochrome c was determined in a double beam spectrophotometer by difference spectroscopy according to fundamental work of Keilin [32]. We used the α band of the cytochrome c spectrum at 550 nm after reduction of the cytochrome by Na-dithionite. Alternatively, cytochrome c was determined by specific antibodies. Mitochondrial sub-fractions corresponding to 5 μg protein were separated by TRICINE 12% Poly-acrylamide gel electrophoresis (PAGE). Contents of cytochrome c were estimated by Western blotting using monoclonal anti-cytochrome c antibody (Pharmingen). Antigen–antibody complexes were detected using a horseradish peroxidase conjugated goat anti-mouse IgG (Sigma) and a chemi-luminescence kit (ECL + Plus, Amersham). A calibration with horse heart cytochrome c was performed. The curve was found to be linear between 5 and 25 pmol cytochrome c.

2.1.7. Assay of protein concentration

Protein was determined by the method of Lowry et al. [33].

2.1.8. Electrophoresis, trans-blot, immune decoration

PAGE was performed according to Laemmli [34] with 100 mM Tris, 100 mM Tricine pH 8.6, 0.1% SDS as electrode buffer. Electro-transfer and immune-decoration were performed as described by Rott and Nelson [35].

2.1.9. Bax-ΔC preparation

Recombinant Bax-ΔC protein used in the experiments shown in Fig. 4 was prepared according to Xie et al. [36] with slight modifications. Briefly, E. coli XL-1 Blue cells carrying the plasmid pGEX-T1-Bax (1–171) were grown overnight at 37 °C in 2 l Terrific Broth (TB) medium supplemented with 1.5% glycerol containing 0.5 mg/ml carbenicillin. Protein overexpression was induced by the addition of 10 μM IPTG and performed overnight at room temperature. The harvested cells were disrupted by lysozyme addition and sonification. After centrifugation (27,500 × g, 10 min) the supernatant was applied to a glutathione sepharose column (Pharmacia) by overnight circulation. After extensive washing, the protein was recovered from the column by proteolytic cleavage with thrombin (Pharmacia). The eluted Bax-ΔC protein was dialyzed for 4 h in the presence of 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, and 0.1% 2-mercaptoethanol. The dialyzed protein was applied to a Resource Q column (Pharmacia) and the protein was eluted within a linear gradient ranging from 0 to 500 mM NaCl in dialysis buffer. Fractions containing the Bax-ΔC protein were concentrated in the presence of 10 mM Tris–HCl (pH 7.5), 100 mM NaCl using a stirred Amicon cell to a final protein concentration of 0.35 mg/ml. The Bax-ΔC protein was stored at −70 °C.

3. Results

3.1. Cytochrome c distribution in mitochondrial sub-fractions

We analysed the distribution of cytochrome c in subfractions of kidney mitochondria obtained by osmotic shock and subsequent sucrose density centrifugation. This method leads to a separation of contact sites from inner membrane fragments and unbroken mitochondria. The contact site fraction is characterised by high hexokinase activity, whereas SDH is representative for all fractions containing inner membrane (Fig. 1A). At low ionic strength, cytochrome c is not detached from the membranes. Thus, in sucrose-based medium used here, even after rupture of the mitochondrial membranes cytochrome c remained bound where it was before disruption. Cytochrome c was determined by immune reaction. The amount of cytochrome c in the different fractions was calculated from the resulting signal intensity. While most of the cytochrome c was found in the SDH activity peak, a significant cytochrome c concentration was present in the hexokinase activity containing fractions, representing contact sites. This suggested that the cyto-
chrome c fraction bound to the surface of the peripheral inner membrane has a specific second localisation at the contact sites.

Contact sites are transient structures that form between functionally interacting components in the outer membrane, for example VDAC and in the peripheral inner membrane, as example ANT. VDAC, interacting with the ANT, has a higher capacity for hexokinase binding compared to free porin beyond the contact sites. Hexokinase forms tetramers in these sites [37,38]. Therefore, the enzyme activity could be used as a marker for this type of contact sites. However, hexokinase binding did not induce contact sites [20] meaning that VDAC–ANT complexes could persist even after desorption of hexokinase. Such detachment of hexokinase was performed with glucose 6-phosphate which changes the structure of hexokinase and by that leads to lower affinity of the enzyme. As depicted in Fig. 1B, hexokinase activity decreased significantly after incubation of mitochondria with glucose 6-phosphate, but the treatment did not reduce}

3.2. Changes of cytochrome c distribution depending on the presence of contact sites

Based on the results above, we concluded that a specific contact site structure might be responsible for cytochrome c binding. Considering that VDAC–ANT complexes are involved in contact site formation we studied the cytochrome c distribution under conditions that changed the structure of the ANT from the c (tractacyloside) to the m (bongkreka) conformation.

Sub-fractions of kidney mitochondria were prepared and separated as described above. Atractyloside known to induce contact sites [39,40] resulted in increased hexokinase, and in parallel, a higher cytochrome c concentration in the contact site fraction (Fig. 2A). However, pre-incubation with bongkreka, which suppresses porin–ANT complexes, lead to a decrease of hexokinase and also of cytochrome c in the contacts (Fig. 2B).
Besides affecting the ANT structure, contacts can be induced by dextran that changes the structure of VDAC [20,41] and facilitates interactions between outer- and peripheral inner membrane by exerting an osmotic effect on the outer compartment. In agreement with these findings, we observed higher activity of hexokinase in the fractions of the density gradient that contained the contact sites Fig. 3A. The opposite result was obtained by depression of the contact sites with glycerol [42]. In this case, only very low activity of hexokinase was found in the contact site fractions (Fig. 3B). Following the cytochrome c distribution in the different mitochondrial sub-fractions, it was observed that the cytochrome c concentration in the contact site fraction was correlated with the hexokinase activity (Figs. 1–3). As bound hexokinase appeared to indicate the presence of VDAC–ANT complexes, it was assumed that cytochrome c was associated to the complexes.

To exclude that this indication was erroneous because of inhibition of hexokinase activity, we analysed the contact sites by electrophoresis and immune decoration under different treatments. The results depicted in Fig. 4 completely agree with that obtained by activity determination. The peptide band in the region of M, 100 kDa, representing the hexokinase monomer, disappeared in the sedimented contacts under glucose-6-P and bongkrekate treatment. Cytochrome c, identified by specific antibodies, was absent in the contact fraction after glycerol and bongkrekate treatment.

### 3.3. The peripheral lysine residues of cytochrome c determine its distribution

It has been described that borate ion interacts with high affinity with the peripheral lysine residues No. 13 and 86 of the cytochrome c [43]. We therefore treated kidney mitochondria with 5 mM Na-borate followed by the same procedure as above of osmotic shock and separation of the sub-fractions by density gradient centrifugation. As shown in Fig. 3C, hexokinase activity was high in the contact site fractions, however, cytochrome c decreases...
while it increased in the cristae-rich fractions of the density gradient.

3.4. Isolation of the hexokinase porin–ANT complex and co-localisation of cytochrome c therein

The results above suggested that cytochrome c may be organised in the VDAC–ANT complexes. Hexokinase arranges as a tetramer at VDAC in this complex [38]. The enzyme can thus be used as a device to isolate the ANT–VDAC complex from a Triton extract of brain membranes [23]. Hexokinase in the Triton extract was bound to an anion exchanger and was eluted by a KCl gradient. Fig. 5 shows the elution profile of hexokinase activity from a DE-cellulose column. When the concentration of cytochrome c was determined in the eluted fractions, we observed a smaller peak running in front of the hexokinase activity, whereas the largest amount was eluted together with the hexokinase. In previous investigations, it has been observed that the hexokinase in these fractions was linked to porin and the ANT. The hexokinase porin–ANT complexes reconstituted in phospholipid vesicles functionally resembled the PT pore [23,24].

Table 1

<table>
<thead>
<tr>
<th>Sample + additions</th>
<th>Malate in supernatant (% of total)</th>
<th>Cytochrome c in supernatant (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>18.6</td>
</tr>
<tr>
<td>50 μM Ca^{2+}</td>
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<td>25.6</td>
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<td>500 μM Ca^{2+}</td>
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<td>16.87</td>
</tr>
<tr>
<td>500 μM Ca^{2+} (100 nM CSA)</td>
<td>30.3</td>
<td>30.78</td>
</tr>
<tr>
<td>Triton X-100 (total)</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 6. Bax-dependent release of endogenous cytochrome c in the hexokinase porin–ANT complex. (A) The vesicles containing reconstituted complexes were loaded with 5 mM malate. Release of the entrapped malate and of endogenous cytochrome c into the supernatant was measured after incubation of the vesicles at 25 °C for 30 min with increasing concentrations of Bax–ΔC in 125 mM sucrose, 10 mM Hepes pH 7. Mean of three experiments with independent complex preparations. (B) The experiment was performed as in A except that in some samples, 250 μM bongkrekate was present. In the samples treated with 250 μM bongkrekate, cytochrome c was determined in the supernatant (BA sup) and sediment (BA sed) after centrifugation. Mean of two experiments with two independent complex preparations. (C) Influence of hexokinase activity on the Bax-dependent release of endogenous cytochrome c. The vesicles containing the reconstituted complexes were incubated at 25 °C for 30 min with increasing concentrations of Bax–ΔC in 125 mM sucrose, 10 mM Hepes pH 7.0. 2 mM ATP and 10 mM glucose were added to the samples. Cytochrome c was determined in supernatant and sediment after centrifugation. Depicted is a typical example of three experiments.
3.5. Bax-dependent cytochrome c release from the reconstituted hexokinase porin–ANT complex

The hexokinase porin–ANT complex was reconstituted in phospholipid vesicles. The vesicles were loaded with malate, which could be released by increasing Ca\(^{2+}\) concentrations. In agreement with earlier investigations [23] the Ca\(^{2+}\)-dependent malate release was inhibited by cyclosporin A (Table 1), suggesting PT pore-like functions in the reconstituted complex.

The same malate-containing vesicles with the reconstituted hexokinase complex were treated with increasing Bax-D concentrations. As depicted in Fig. 6A, Bax-D was able to liberate the cytochrome c bound in the complex in a concentration range between 400 and 1000 nM. However, the malate inside the vesicles was not released.

The Bax-dependent cytochrome c release could be completely suppressed by incubation of the reconstituted complexes with 250 μM bongkrekate (Fig. 6B). Considering that bongkrekate reduced contact sites and by that changed the cytochrome c distribution (Fig. 2B), the results suggested that Bax-D at the applied low concentrations liberated exclusively a fraction of cytochrome c that was organised within VDAC–ANT complexes constituting contact sites.

3.6. Hexokinase activity suppresses Bax-Dc-dependent cytochrome c release

It has been observed that Bax and hexokinase isotype II compete for the same binding sites [25]. The ADP produced by active hexokinase stabilises the porin–ANT complexes [39], and thus the enzyme stays bound to the mitochondrial surface. This may explain why Bax-D was unable to displace hexokinase and to liberate cytochrome c from the reconstituted hexokinase porin–ANT complex in the presence of hexokinase substrates, glucose and ATP (Fig. 6C).

4. Discussion

4.1. Localisation of cytochrome c in mitochondria

Considering the new mitochondrial structure model [15,16], at least two fractions of cytochrome c were expected: one fraction at the surface of the cristae membranes and the other bound to the peripheral inner membrane. Here we show that a third fraction of cytochrome c has to be regarded in the contact sites. However, the cytochrome c localisation in the contact sites varied, depending on the presence or absence of VDAC–ANT complexes (Figs. 1–3). The outer membrane pore VDAC adopts a different structure when it forms a complex with the ANT. This VDAC structure has a higher capacity for binding of hexokinase isotype I [20] and, indicated the presence of complexes with ANT. The hexokinase type I isozyme is the predominant enzyme in brain and kidney cortex that remains tightly attached to the mitochondrial fractions [44]. Thus, hexokinase type I could be used to isolate the contact site complexes from Triton X-100 extracts of brain or kidney cortex membranes [40]. By changing the structures of VDAC by dextran (Fig. 3A), or of the ANT by atractyloside and bongkrekate (Fig. 2A,B) or of hexokinase by glucose 6-phosphate (Fig. 1B), it was found that the concentration of cytochrome c in the contact site fraction was depending on the existence of VDAC–ANT complexes. The well-known pro-apoptotic effect of atractyloside and the anti-apoptotic function of bongkrekate can be explained by altering the VDAC–ANT complexes followed by changes of the cytochrome c distribution. In agreement with this, it was recently observed that the anti-apoptotic Bcl-2 suppresses induction of contact sites [45].

4.2. Cytochrome c is a component of the isolated VDAC–ANT complexes

The results above suggested that cytochrome c might be a component of the hexokinase VDAC–ANT complexes that provide the structure of one type of contact sites. In earlier investigations, it was found that the complete VDAC–ANT complex was stable in Triton X-100 extracts of brain and kidney membranes [23,40]. When such hexokinase contact sites were isolated from brain membrane extracts, we observed that cytochrome c was also a component of the isolated VDAC–ANT complexes and endured the isolation procedure (Fig. 5).

Neither the nature of the cytochrome c organisation inside contact sites nor the function of cytochrome c in the porin–ANT complex is known so far. It can only be speculated that the negatively charged phospholipid cardiolipin that is associated with the ANT [46] may provide the binding site for the positively charged cytochrome c. In support of this assumption, we observed that treatment with Na-borate caused a decrease of cytochrome c in the contact site fraction. According to the proposal of Taler et al. [43], the borate ion reacts with the peripheral lysines of cytochrome c. High ionic strength of 200 mM KCl was applied to elute the hexokinase porin–ANT complex from the anion exchanger column, but the cytochrome c remained bound to the complex (Fig. 5). Cortese et al. [47] already described such a cytochrome fraction in mitochondria that was firmly bound and resistant to extraction at high ionic strength. This membrane-bound fraction increased when the volume of the inter-membrane compartment was decreased. The authors found that the membrane-bound cytochrome c fraction readily exchanged with externally added cytochrome c. The same phenomenon was recently observed as a function of Bax in cardiomyocytes and isolated heart mitochondria. Bax-Dc released 10% of the total cytochrome c resulting in 30% depression of respiration that could be completely restored by addition of external cytochrome c [48].

We propose that a special organisation of cytochrome c in the contact sites or contact site-derived porin–ANT
complexes is required to cause its release by Bax-ΔC. This organisation could involve partial integration of cytochrome c into the membrane by binding to cardiolipin that might distribute along the hydrophobic surface of the porin–ANT complex. This organisation may thus not need pore formation for the releasing process. Indeed, a movement of cardiolipin to the outer leaflet of inner membrane has been observed preceding apoptosis [49].

4.3. Bax released endogenous cytochrome c from the reconstituted VDAC–ANT complex without pore formation

The hexokinase porin–ANT complexes were reconstituted in phospholipid vesicles as described recently [24]. Bax was able to release the cytochrome c originally bound in the complex, but malate, which was loaded into the same vesicles, was not liberated (Fig. 6A). However, the malate could be released from the same vesicles through the PT pore (presumably the ANT), by addition of Ca²⁺ (Table 1). Thus, it is concluded that Bax-ΔC in the range between 400 and 1000 nM did not form pores but detached cytochrome c from specific binding sites in the porin and ANT complexes.

4.4. Bax binds to hexokinase binding sites

Recently Pastorino et al. [25] described a competition between hexokinase and Bax for the binding sites at the mitochondrial surface. It thus appeared that Bax, such as hexokinase, might have preference to bind to the VDAC interacting with the ANT. The dissociation of the porin–ANT complex by bongkrekate firstly changes the distribution of cytochrome c and secondly decreases the affinity of hexokinase and presumably Bax to VDAC (Fig. 2B). Because of these two reasons, bongkrekate suppressed release of the endogenous cytochrome c from the reconstituted complexes (Fig. 6B).

4.5. Hexokinase activity inhibits Bax-dependent cytochrome c release in reconstituted porin–ANT complexes

The ADP produced by active hexokinase stabilises the VDAC–ANT complexes [39] and thus the enzyme stays bound to the mitochondrial surface. This may explain why Bax-ΔC was unable to displace hexokinase and to liberate cytochrome c from the reconstituted hexokinase porin–ANT complex in the presence of hexokinase substrates glucose and ATP (Fig. 6C). Several recently published observations in intact cells are explicated by our finding. It has been described that constitutively active protein kinase B/Akt-inhibited growth factor withdrawal induced apoptosis by increased rate of glycolysis [27,50]. Active protein kinase B was found to increase mitochondrial binding of hexokinase II in Rat 1a cells and suppressed cytochrome c release and apoptosis through activity of hexokinase in the presence of glucose [26]. In agreement with this, B-cell lines derived from patients with Hodgkin’s disease, known to have high mitochondrial bound hexokinase activity [51], were resistant against staurosporin-induced apoptosis [52].

All these results strongly suggest that Bax-ΔC can specifically release a fraction of mitochondrial cytochrome c that is organised in a so far unknown way close to or within the VDAC–ANT complexes. This portion of cytochrome c may be a physiologically fast responding fraction observed in the early phase of Bax action [9,28]. As this fraction amounts to maximally 20% of the mitochondrial cytochrome c, the Bax-ΔC-dependent release did not block the electron transport and mitochondrial function [48]. It has been observed that Bcl-2 and Bcl-xl interact as well with the reconstituted hexokinase complex and inhibited the PT pore opening [53]. It was recently described that Bcl-2 suppressed contact sites [45], suggesting that by this mechanism, Bcl-2 may interfere with the Bax effect on cytochrome c release.

On the whole, our results propose that activity of hexokinase bound to the VDAC–ANT complex can inhibit the release of both cytochrome c fractions: the Bax-induced release through interactions with the contact sites, and the massive release of cytochrome c by swelling and disruption of the membranes through Ca²⁺-dependent opening of the PT pore.

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