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Hexokinase Receptor Complex in Hepatoma Mitochondria: Evidence from N,N'-Dicyclohexylcarbodiimide-Labeling Studies for the Involvement of the Pore-Forming Protein VDAC[†]

Richard A. Nakashima,[‡] Patrick S. Mangan,[§] Marco Colombini,[§] and Peter L. Pedersen[*,‡]

Laboratory for Molecular and Cellular Bioenergetics, Department of Biological Chemistry, School of Medicine, The Johns Hopkins University, Baltimore, Maryland 21205, and Laboratories of Cell Biology, Department of Zoology, University of Maryland, College Park, Maryland 20742

Received July 24, 1985

ABSTRACT: In rapidly growing, highly glycolytic hepatoma cells as much as 65% of the total cell hexokinase is bound to the outer mitochondrial membrane [Parry, D. M., & Pedersen, P. L. (1983) *J. Biol. Chem.* *258,* 10904–10912]. In this paper, we describe the purification to apparent homogeneity of a mitochondrial pore-forming protein from the highly glycolytic AS-30D rat hepatoma cell line. The purified protein shows a single 35 000-dalton band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, an amino acid composition slightly more hydrophobic than that of the rat liver pore protein (also known as VDAC or mitochondrial porin), and a channel-forming activity of 136 channels min^{-1} (μg of protein)$^{-1}$. In addition to displaying the properties characteristic of VDAC (single-channel conductance, voltage dependence, and preference for anions), we observe that the AS-30D VDAC protein is one of only three mitochondrial proteins that bind [^{14}C]dicyclohexylcarbodiimide (DCCD) at relatively low dosages (2 nmol of DCCD/mg of mitochondrial protein). Significantly, treatment of intact mitochondria isolated from either rat liver or the AS-30D hepatoma with DCCD results in an almost complete inhibition of their ability to binding hexokinase. Fifty percent inhibition of binding occurs at less than 2 nmol of DCCD/mg of mitochondrial protein. In contrast to DCCD, water-soluble carbodiimides are without effect on hexokinase binding. These results suggest that the pore-forming protein of tumor mitochondria forms at least part of the hexokinase receptor complex. In addition, they indicate that a carboxyl residue located within a hydrophobic region of the receptor complex may play a critical role in hexokinase binding.

Oने of the most consistent phenotypic markers of transformed cell lines is an increase in glucose utilization and lactic acid production rates compared to normal cells from the same tissue of origin (Warburg et al., 1924; Cori & Cori, 1925; Weinhouse, 1966; Burk et al., 1967; Bustamante et al., 1981). The increased rates of glucose catabolism promote rapid tumor growth by providing an alternate source of ATP production, accounting for up to 60% of total cell ATP production in highly glycolytic cancer cells (Aisenberg, 1961; Pedersen, 1978; Nakashima et al., 1984), and by increasing intracellular levels of glucose-6-phosphate, a required precursor in the de novo synthesis of nucleic acids and other essential macromolecules (Weber, 1977). One of the factors that appears to be related to increased glycolytic activity in tumor cells is an increase in total activity and a change in the subcellular distribution of hexokinase (EC 2.7.1.1), which occurs as a predominantly mitochondrially bound enzyme in rapidly growing transformed cell lines (Rose & Warms, 1967; Bustamante & Pedersen, 1977). Binding of hexokinase to the mitochondrial outer membrane has been reported to result in a loss of feedback inhibition by the product glucose 6-phosphate (Kosow & Rose, 1968; Gumaa & McLean, 1969; Wilson, 1968; Bustamante & Pedersen, 1977; Singh et al., 1974) and in a preferred access to mitochondrially generated ATP (Gumaa & McLean, 1969; Gots & Bessman, 1974; Inui & Ishibashi, 1979). Previous studies in our laboratory have shown that the elevated levels of mitochondrially bound hexokinase are essential for the high rates of glycolysis observed in rapidly growing cancer cell lines (Bustamante & Pedersen, 1977; Bustamante et al., 1981).

An outer membrane hexokinase receptor site appears to be of wide distribution in mitochondria, even in those tissues that apparently exhibit little hexokinase binding in vivo (Rose & Warms, 1967; Parry & Pedersen, 1983). Felgner et al. (1979) were the first to isolate a hexokinase binding protein from the outer mitochondrial membrane of rat liver. This integral membrane protein of apparent M_r 31 000–32 000 was shown by Linden et al. (1982a) and Fiek et al. (1982) to be identical with the outer membrane pore-forming protein (also known as VDAC[1] or mitochondrial porin) of rat liver mitochondria.

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[1] Abbreviations: VDAC, voltage-dependent, anion-selective, channel-forming protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DCCD, N,N'-dicyclohexylcarbodiimide; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; HPLC, high-pressure liquid chromatography; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

Despite an abundance of studies on the mitochondrial pore-forming protein of normal eukaryotic cell lines [for review, see Colombini (1980)], neither the outer membrane pore protein nor the hexokinase receptor site has been characterized in mitochondria of a transformed cell line. Given the apparent involvement of mitochondrial hexokinase in the maintenance of the high glucose utilization rates of rapidly growing cancer cells, it seemed important to confirm that the pore-forming protein is involved in hexokinase binding to tumor mitochondria and to compare the characteristics of this protein in normal and transformed cell lines.

The purposes of this study were 3-fold, to purify and characterize the outer membrane pore protein from the rapidly growing AS-30D rat hepatoma cell line and compare it to the normal rat liver protein, to determine whether or not the outer membrane pore protein is involved in hexokinase binding to tumor mitochondria, and to examine the effects of the hydrophobic reagent N,N'-dicyclohexylcarbodiimide (DCCD) on mitochondrial binding of hexokinase. In a recent paper, De Pinto et al. (1985) showed that DCCD covalently labeled the outer membrane pore protein of pig heart mitochondria at low dosages. It thus seemed possible that DCCD might affect mitochondrial hexokinase binding as well. The results presented demonstrate that DCCD is an effective inhibitor of mitochondrial hexokinase binding at relatively low dosages and that the pore-forming protein appears to form at least part of the hexokinase receptor complex in tumor mitochondria.

EXPERIMENTAL PROCEDURES

Chemicals. Hydroxylapatite (Bio-Gel HTP) was obtained from Bio-Rad Laboratories. DEAE- and CM-Sepharose were from Pharmacia. DCCD was obtained from the Schwarz/Mann Division of Becton, Dickinson and Co. Aquacide III was from Calbiochem-Behring. En³Hance was obtained from New England Nuclear. [¹⁴C]DCCD and Triton X-100 were purchased from Research Products International Corp. Lubrol WX was obtained from the Grand Island Biological Co. The nonionic detergent Genapol X-80 was the generous gift of the American Hoechst Corp. of Somerville, NJ. (It is currently available from Calbiochem-Behring.) Genapol X-80 resembles Triton X-100 in containing polyethoxy chains. However, the central aromatic ring of Triton X-100 has been replaced in Genapol X-80 by an isotridecanol group, which does not absorb at 280 nm. Asolectin (soybean phospholipids) was purchased from Sigma Chemical Co. and purified by the method of Kagawa and Racker (1971).

Animals and Tumor Cells. The AS-30D rat hepatoma cell line was obtained from Dr. A. L. Lehninger of the Johns Hopkins University School of Medicine, Baltimore, MD. The line was maintained in ascites form by serial intraperitoneal injection into 100–150-g female Sprague-Dawley rats purchased from Holtzman Laboratories (Madison, WI). AS-30D cells were collected and purified as described by Parry and Pedersen (1983).

Mitochondrial Isolation. Tumor mitochondria were isolated from purified AS-30D cells by the sonication technique of Parry and Pedersen (1983). Rat liver mitochondria were isolated by the high-yield method of Bustamante et al. (1977). In both cases after the cell homogenization step, the mitochondria were resuspended in H medium (210 mM D-mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.4) without BSA or EGTA added. Mitochondria were either used fresh or else frozen in liquid nitrogen and thawed immediately prior to use.

Tumor Hexokinase. Purified AS-30D mitochondria (5–10 mg/mL) were suspended in H medium with 1 mM PMSF,

1 mM EGTA, and 1 mM DTT added to inhibit protease activity. After addition of 1.6 mM ATP, the mitochondria were incubated for 15 min at 30 °C. This resulted in the selective solubilization of mitochondrially bound hexokinase (Bustamante & Pedersen, 1980). Following centrifugation of the suspension at 20000g for 15 min, the resulting supernatant was centrifuged at 130000g for 45 min to remove mitochondria and particulate contaminants. This procedure resulted in the substantial purification of tumor hexokinase with a specific activity between 20 and 60 units/mg. Hexokinase activity was determined by standard spectrophotometric assay as described previously (Parry & Pedersen, 1983).

Hexokinase Binding Assay. Solubilized tumor hexokinase isolated from AS-30D mitochondria by the protocol described above was used in all assays for hexokinase binding activity. Purified mitochondria (2.0 mg/mL) from either rat liver or AS-30D hepatoma were incubated with tumor hexokinase (1–5 units/mL) in 0.5 mL of H medium supplemented with 10 mM MgCl₂. After 15 min at room temperature, the suspension was diluted with 0.9 mL of ice-cold H medium and centrifuged for 1.5 min at 12800 g in an Eppendorf centrifuge. The mitochondrial pellets were resuspended in H medium, and aliquots were solubilized with the nonionic detergent Lubrol WX prior to assay for bound hexokinase activity. AS-30D mitochondria were pretreated with ATP to remove endogenous bound hexokinase prior to the binding assay. Since this treatment did not remove all of the bound hexokinase, duplicate aliquots of AS-30D mitochondria were incubated in the absence of added hexokinase and assayed for bound hexokinase activity as described above. The amount of hexokinase bound to these control mitochondria (approximately 300 milliunits/mg) was subtracted from the experimental values to obtain the amount of added hexokinase that bound to the hepatoma mitochondria. Freshly isolated rat liver mitochondria did not contain any detectable endogenous bound hexokinase activity.

DCCD Pretreatment of Mitochondria. Mitochondria (5–20 mg/mL) suspended in H medium were incubated at room temperature with the dosages of DCCD indicated in Figure 3. After 15 min, the suspension was diluted with ice-cold H medium containing 5 mM MgCl₂ and 1 mg/mL BSA and centrifuged for 10 min at 20000g. The pellet was washed once in H medium and centrifuged again for 15 min at 20000g. After resuspension in H medium, the DCCD-pretreated mitochondria were assayed for hexokinase binding activity as described above. Control mitochondria pretreated in an identical manner with solvent (methanol) alone were used to determine the 100% level of hexokinase binding, which was then used to normalize the hexokinase binding data for the DCCD-pretreated mitochondria. The same conditions of incubation and washing were used in experiments in which the hexokinase receptor protein was labeled with [¹⁴C]DCCD in intact mitochondria.

Purification of the Mitochondrial VDAC Protein from AS-30D Hepatoma Cells. Purified AS-30D mitochondria were extracted with 2% Triton X-100 in 10 mM Tris-HCl, pH 7.0, for 60 min. After centrifugation at 147000g for 45 min, the supernatant was discarded, and the pellet was extracted with 2.5% Genapol X-80 in 50 mM KCl, 10 mM potassium phosphate, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.0, for 60 min. Following centrifugation at 147000g for 30 min, the supernatant containing pore-forming protein was added to Pasteur pipets filled with dry hydroxylapatite. Upon elution with buffered 2.5% Genapol X-80, the pore protein was collected in the initial fractions after the void volume. The

eluate was dialyzed overnight against 2 × 2 L of 0.05% Genapol X-80 and 10 mM Tris-HCl, pH 7.0, and then chromatographed on DEAE-Sepharose and CM-Sepharose (1 × 11 cm columns). The columns were eluted with 0.05% Genapol X-80 buffered with 10 mM Tris-HCl or 10 mM sodium phosphate, respectively, at pH 7.0. The pore-forming protein was not retained by either column and was collected from the first fractions after the void volume. Since Genapol X-80 does not absorb substantially at 280 nm, we used OD$_{280}$ to determine the pore protein containing fractions. The final eluate was concentrated in dialysis tubing coated with Aquacide III. All isolation steps were performed at 4 °C.

Assay of VDAC Activity. In order to demonstrate that the protein purified from the hepatoma cells was VDAC, it was assayed for channel-forming activity as previously described (Colombini, 1979; Schein et al., 1976). A planar lipid (asolectin) bilayer was made by the method of Montal and Mueller (1972) across a 0.14-mm hole in a Saran partition separating two Teflon compartments, each of about 4-mL capacity. Channel insertions were observed upon addition of the purified protein solution to the aqueous phase on one side of the bilayer (1 M KCl and 5 mM CaCl$_2$ on each side of the membrane). These insertions were observed as stepwise increases in the ion permeability of the membrane measured as current increments in response to a 10-mV driving force [the results have been converted to conductance increments (see Figure 1)]. The voltage across the membrane was controlled by means of a voltage clamp similar to that described previously (Schein et al., 1976). Control solutions (buffered detergent solutions without protein or with cytochrome c or bovine serum albumin) produced no increase in membrane ion permeability when added as described above.

The insertion of channels into a phospholipid membrane was observed when the pure protein was used or when crude fractions were used. The rate of channel insertion into the membrane was used as a measure of channel-forming activity [as done previously (Colombini, 1979, 1983)] in the various fractions at different stages in the purification protocol described above.

Other Techniques. SDS–polyacrylamide gel electrophoresis was carried out according to Laemmli (1970) with 14% acrylamide gels. [^{14}C]DCCD-labeled bands on polyacrylamide gels were identified by fluorography (Bonner & Laskey, 1974) after treatment of the gel with En^{3}Hance. Protein concentration was measured by the biuret technique (Jacobs et al., 1956) in the case of mitochondria and by the Lowry assay (Lowry et al., 1951) for purified proteins in the presence of detergent. The amino acid composition of purified AS-30D VDAC was kindly determined by Dr. Mary P. Strickler and Mary J. Gemski of Waters Associates, Inc., of Rockville, MD.

RESULTS

Purification of the VDAC Protein from AS-30D Hepatoma Cells. The VDAC protein was purified from whole AS-30D mitochondria by a modification of two previously published techniques (Linden et al., 1982b; Freitag et al., 1982). This modification was found to be necessary when whole hepatoma mitochondria were used as the starting material as opposed to purified rat liver outer mitochondrial membrane (Linden et al., 1982b) or *Neurospora crassa* mitochondria (Freitag et al., 1982). The details of the purification protocol used in this study are given under Experimental Procedures. The relative purification of the AS-30D pore-forming protein was followed by measuring the specific activity for VDAC at various stages of the purification process (data shown in Table I). The amount of activity present in each fraction was determined

Table I: Scheme for VDAC Purification from AS-30D Mitochondria[a]

fractions	initial protein remaining (%)	VDAC sp act. (channels min^{-1} μg^{-1})	purification (x-fold)
AS-30D mitochondria	100	1.4[b]	1
Genapol X-80 extract	10	5	4
hydroxylapatite eluate	0.7	52	37
DEAE-Sepharose eluate	0.2	97	69
CM-Sepharose eluate	0.08	136	97

[a] VDAC was purified from whole AS-30D mitochondria by the protocol described under Experimental Procedures. At the various stages of the purification process, aliquots of partially purified pore-forming protein were assayed for VDAC activity as described in the legend to Figure 1. The slope of the initial insertion phase after protein addition was measured, and the VDAC specific activities were calculated as channels inserted into the bilayer per minute per microgram of protein added. It should be noted that the specific activities derived by this technique are a qualitative rather than quantitative measure of pore-forming activity. The data reported represent the averaged values for two preparations of AS-30D VDAC. The recovery of protein is reported here as the percent of initial mitochondrial protein remaining. [b] Specific activity present in Triton X-100 extract of AS-30D mitochondria.

by measuring the unit channel insertion rate [as described by Colombini (1979)]. The data indicate a 97-fold purification of VDAC activity. The specific activity present in the final fraction [136 channels min^{-1} (μg of protein)$^{-1}$] is within the range for VDAC specific activity reported by Colombini (1983) for the outer membrane protein purified from normal rat liver mitochondria. The total amount of VDAC activity in the final fraction represents approximately a 10% recovery of initial activity. Protein recovery is reported as percent of initial mitochondrial protein. Starting with 20 mg of whole mitochondrial protein, we obtain approximately 16 μg of purified AS-30D VDAC.

Figure 1 shows a typical recording of VDAC activity for the hepatoma protein. Following addition of protein (at the arrow), a stepwise increase in conductance is observed, which represents the insertion of ion-permeable channels into the phospholipid membrane. The most probable conductance of an individual insertion was 4.0 nS, which is very close to values reported for rat liver VDAC (Colombini, 1979, 1983; Roos et al., 1982). This value varied over a narrow range, indicating that the channels are homogeneous. For these channels to be VDAC, they must be voltage-dependent. The insert to Figure 1 shows channel closure at 40 mV. When the voltage was raised from 10 to 40 mV, the current at first increased 4-fold due to the 4-fold greater driving force, but soon afterward, it decayed as channels closed. When the voltage was reduced to 10 mV, once again the current returned to its original value, indicating that the channels had reopened very rapidly. The slow kinetics of closure and the rapid opening kinetics are also characteristic of VDAC (Colombini, 1979).

The selectivity of the channels was determined by inserting the channels into a membrane in the presence of a transmembrane salt gradient (1 M KCl and 5 mM CaCl$_2$ on one side and 0.1 M KCl and 5 mM CaCl$_2$ on the other). The current observed in the absence of an electric field indicated a preference for Cl$^-$. The voltage needed to bring this current to zero (the reversal potential) was 11 mV. This is the same value as reported for rat liver VDAC (Colombini, 1983) and is consistent with a 2-fold selectivity of Cl$^-$ over K$^+$.

Analysis of Purified AS-30D VDAC by SDS–Polyacrylamide Gel Electrophoresis. The polypeptide composition of AS-30D VDAC was analyzed by SDS–PAGE according to Laemmli (1970) in a 14% acrylamide slab gel. As shown in
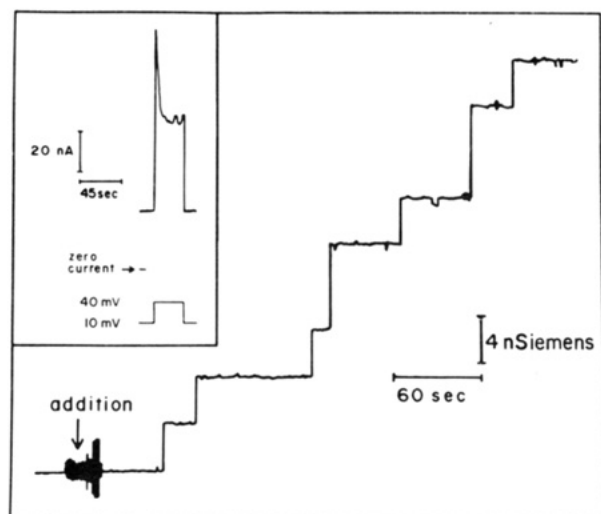
FIGURE 1: Properties of the channels formed by the AS-30D protein. At the point indicated by the arrow, 10 μL of a diluted solution containing Genapol X-80 solubilized AS-30D protein were added to the aqueous phase bathing a planar phospholipid membrane [made by the Montal-Mueller technique as previously described (Schein et al., 1976)]. Both sides of the membrane were in contact with 1.0 M KCl and 5 mM CaCl₂ solutions. After a short time, the conductance of the membrane (in nanosiemens) increased in a stepwise fashion characteristic of channels. The inset shows the voltage-dependence of these channels for a membrane containing about 200 channels.

Table II: Amino Acid Composition of Purified AS-30D VDAC[a]

amino acid	composition of AS-30D VDAC (mol %)	composition of rat liver VDAC (mol %)
polar		
Lys	7.7	9.4
His	1.1	1.4
Arg	4.8	2.3
Asx	6.4	11.6
Thr	9.1	9.0
Ser	6.9	5.8
Glx	8.6	8.3
nonpolar		
Pro	3.6	2.5
Gly	8.9	11.7
Ala	8.0	7.4
Cys	UD[b]	ND[c]
Val	6.6	5.9
Met	UD	0.6
Ile	4.8	3.7
Leu	13.2	10.0
Tyr	4.0	4.4
Phe	6.4	6.2
Trp	ND	ND

[a] VDAC was purified from whole AS-30D mitochondria as described under Experimental Procedures. The purified protein was hydrolyzed overnight with 6 N HCl at 110 °C, and the phenylthiocarbamyl-derivatized amino acid residues were analyzed by HPLC on a Waters Pico-Tag column. The analysis was performed by Dr. Mary P. Strickler and Mary J. Gemski of Waters Associates, Inc. For comparison, the amino acid composition of pore-forming protein isolated from rat liver outer mitochondrial membrane is also included [from Linden et al. (1982b)]. [b] Undetectable. [c] Not determined.

the inset to Figure 2, when 10 μg of the purified AS-30D VDAC was run on a gel and stained with Coomassie brilliant blue R-250 (Bio-Rad), it migrated as a single polypeptide species of apparent M_r 35 000 in this gel system. Its apparent molecular weight is within the range of previously reported values (30 000–35 000) for VDAC isolated from mammalian mitochondria (Zalman et al., 1980; Linden et al., 1982b; Roos et al., 1982; De Pinto et al., 1985). When the purified VDAC proteins of AS-30D hepatoma and rat liver mitochondria were analyzed in adjacent lanes on an SDS–polyacrylamide slab
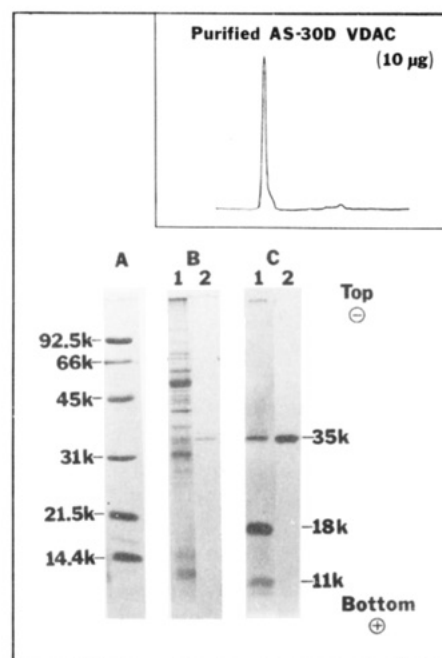

FIGURE 2: [¹⁴C]DCCD labeling of whole AS-30D mitochondria. Freshly isolated AS-30D hepatoma mitochondria were incubated with [¹⁴C]DCCD at 2 nmol/mg of protein and then washed with H medium containing 1 mg/mL BSA. The [¹⁴C]DCCD-treated mitochondria were sequentially extracted with 2% Triton X-100 and 2.5% Genapol X-80, and AS-30D VDAC was purified from the Genapol X-80 extract as described under Experimental Procedures. Aliquots containing 30 μg of Triton X-100 extract (B and C, lanes 1) and 1.5 μg of VDAC (B and C, lanes 2) were run on a 14% polyacrylamide gel in the presence of SDS. (A) The molecular weight markers (obtained from Bio-Rad) were phosphorylase B (92 500), BSA (66 200), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 400). (B) The gel containing the Triton X-100 extract (lane 1) and AS-30D VDAC (lane 2) was stained with Coomassie brilliant blue R-250 (Bio-Rad) to visualize the peptide composition. (C) The Coomassie-stained gel was impregnated with En³Hance (New England Nuclear) and dried. The [¹⁴C]DCCD-labeled bands were localized by fluorography with Kodak XAR-5 X-ray film. Aside from material that did not enter the gel (lane 1, top) only three DCCD-labeled bands were present in the Triton X-100 mitochondrial extract (C, lane 1) of apparent M_r 35 000, 18 000, and 11 000. A very faint additional band can be seen in the Triton X-100 extract (C, lane 1, bottom), which migrated close to the solvent front and may consist with DCCD-labeled membrane lipids. Purification of the VDAC protein from [¹⁴C]DCCD-labeled mitochondria resulted in the substantial enrichment of the 35 000-dalton DCCD binding protein (C, lane 2). The inset shows an optical scan of a 10-μg sample of AS-30D VDAC analyzed by SDS–PAGE and stained with Coomassie brilliant blue R-250.

gel, they migrated with equal electrophoretic mobility (data not shown).

Amino Acid Composition of Purified AS-30D VDAC Protein. The amino acid composition of AS-30D VDAC was determined according to Bidlingmeyer et al. (1984) by high-pressure liquid chromatography of the phenylthiocarbamyl-derivitized amino acid residues following acid hydrolysis of the purified protein. The results of this analysis are presented in Table II. For purpose of comparison, the reported amino acid composition (Linden et al., 1982b) of the rat liver outer mitochondrial membrane pore-forming protein is also shown in Table II. The overall composition seems to be relatively similar for the two proteins. However, the hepatoma VDAC appears to have a higher content of nonpolar residues in comparison with the rat liver pore protein. Calculation of the polarity index according to Capaldi and Vanderkooi (1972) results in a value of 44.6% polar amino acid residues for the AS-30D VDAC protein vs. 47.8% for the rat liver pore protein.

Both of these are in the high range of values for intrinsic membrane proteins (Capaldi & Vanderkooi, 1972). However, considering that the pore protein forms a hydrophilic channel through the membrane, the presence of a high content of polar amino acid residues should not be considered unusual. It should be noted that Gln and Asn are converted to Glu and Asp during the procedure used to obtained the amino acid composition.

Binding of [^{14}C]DCCD to the AS-30D VDAC. The hydrophobic reagent DCCD is known to act as a covalent inhibitor of a number of mitochondrial membrane proteins, including the F_1F_0 ATPase (Cattell et al., 1971), the cytochrome $b-c_1$ complex (Beattie & Villalobo, 1982), the potassium–proton antiporter (Martin et al., 1984), and cytochrome c oxidase (Casey et al., 1980). In a recent paper (De Pinto et al., 1985), it was suggested that DCCD might also covalently bind to the outer membrane pore-forming protein of porcine heart mitochondria. The results presented in Figure 2 show that the VDAC protein of AS-30D mitochondria is labeled with DCCD at low dosages of this reagent. When intact AS-30D mitochondria were treated with [^{14}C]DCCD at 2 nmol of DCCD/mg of mitochondrial protein and washed with BSA, only three DCCD-labeled peptide bands were observed in a subsequent Triton X-100 mitochondrial extract (Figure 2C, lane 1), with relative electrophoretic mobilities of approximately 35 000, 18 000, and 11 000. Purification of VDAC from hepatoma mitochondria pretreated with [^{14}C]-DCCD resulted in the selective enrichment of the DCCD-labeled band of M_r 35 000 (Figure 2C, lane 2). This DCCD-labeled fraction exhibited VDAC activity in the standard assay system (data not shown). It would appear from these results that the AS-30D mitochondrial VDAC is selectively labeled (along with two other peptides) at low dosages of DCCD.

DCCD Inhibition of Hexokinase Binding to Intact Mitochondria. In consideration of previous suggestions that the pore-forming protein and the hexokinase receptor protein of rat liver mitochondria may be identical (Fiek et al., 1982; Linden et al., 1982a), we decided to examine the effects of DCCD treatment on mitochondrial binding of hexokinase. When either rat liver mitochondria or AS-30D hepatoma mitochondria were pretreated with DCCD, washed with BSA, and then examined for hexokinase binding activity, we observed a dose-dependent inhibition of hexokinase binding (data shown in Figure 3). The dose–response curves for DCCD inhibition of binding activity were similar for hepatoma and rat liver mitochondria, with the 50% level of inhibition reached at less than 2 nmol of DCCD/mg of mitochondrial protein. In agreement with previous studies on DCCD inhibition of membrane proteins (Solioz, 1984), addition of the hydrophilic carboxyl group modifier 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDAC) or N-ethyl-5-phenyl-isoxazolium-3'-sulfonate (Woodward's Reagent K) had no significant effect on mitochondrial hexokinase binding at dosages of up to 100 nmol/mg of mitochondrial protein (Table III). Treatment of rat liver mitochondria with the covalent sulfhydryl modifying reagent N-ethylmaleimide also produced no inhibition of hexokinase binding when assayed under the conditions used with DCCD (Table III).

DISCUSSION

The data presented in this paper contain several novel results of potential significance to understanding the abnormal energy metabolism of neoplastic tissues. First, we provide a method for the purification of a mitochondrial VDAC protein (porin) from a transformed cell line, the AS-30D rat hepatoma (Figure 1 and Table I). Second, we report the first evidence for a
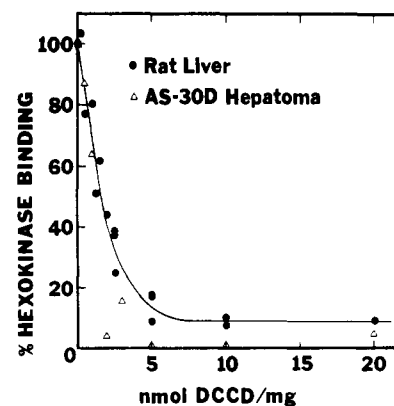

FIGURE 3: Effect of DCCD pretreatment on mitochondrial binding of hexokinase. Whole mitochondria suspended in H medium were treated for 15 min at room temperature with the amounts of DCCD indicated. after being washed with BSA-containing medium, the mitochondria were resuspended in fresh H medium, and their ability to bind solubilized AS-30D hexokinase was assayed as described under Experimental Procedures. Control mitochondria treated with solvent (methanol) alone were used to determine the 100% level of hexokinase binding. The data are plotted as relative percent hexokinase binding vs. the dosage of DCCD and are based upon three experiments with rat liver mitochondria (●) and one experiment with AS-30D mitochondria (△). Under the conditions of these experiments, the 100% level of binding corresponded to 110, 170, and 112 milliunits/mg for rat liver and 232 milliunits/mg for AS-30D mitochondria. AS-30D mitochondria were depleted of endogenous bound hexokinase activity by incubation with 1.6 mM ATP prior to the binding assay. Since the ATP treatment did not remove all of the bound hexokinase, the amount of bound hexokinase activity present at the end of the binding assay was corrected by subtracting the amount of hexokinase bound to duplicate aliquots of DCCD-treated mitochondria incubated without any added hexokinase (approximately 300 milliunits/mg).

Table III: Effects of Carboxyl and Sulfhydryl Modifiers on Hexokinase Binding[a]

reagent	dosage (nmol/mg)	hexokinase binding (%)
DCCD	2	39
	20	9
EDAC	2	109
	20	97
	100	82
Woodward's Reagent	2	102
	10	92
	100	90
N-ethylmaleimide	50	118

[a] Rat liver mitochondria (10 mg/mL) were incubated with the indicated dosages of reagents in 1 mL of H medium for 15 min at room temperature. Following incubation, the suspensions were diluted with 2 mL of ice-cold H medium containing 5 mM $MgCl_2$ and 1 mg/mL of BSA. After centrifugation for 15 min at 20000g, the pellets were washed once with H medium and then resuspended and assayed for hexokinase binding activity as described under Experimental Procedures. Data are reported as percent of binding relative to control mitochondria treated with solvent alone. DCCD was in 95% ethanol, and all other reagents were in distilled water.

selective covalent inhibition of mitochondrial hexokinase binding by the hydrophobic reagent DCCD (Figure 3). Finally, we report that DCCD binds to the hepatoma VDAC at relatively low dosages, at which hexokinase binding is also inhibited (Figures 2 and 3). Taken together, these results indicate that the hepatoma VDAC that we have purified to apparent homogeneity (inset to Figure 2) is involved in the binding of hexokinase to the outer mitochondrial membrane. On the basis of the rate at which these channels insert into phospholipid membranes (Table I), the final protein fraction represents a 97-fold purification of VDAC activity.

A comparison of amino acid composition and channel characteristics between the pore-forming proteins of hepatoma

AS-30D and normal rat liver mitochondria indicates that the two proteins are structurally and functionally similar, but not identical (Table II). The effects of the differences in composition on pore-forming function and hexokinase binding activity are unknown at present. However, it is possible that the difference in composition may be relevant to the great increase in mitochondrial binding of hexokinase observed in rapidly growing cancer cells (Bustamante et al., 1981; Parry & Pedersen, 1983).

Our interest in the hepatoma VDAC or "porin" is primarily related to its possible role in mitochondrial binding of hexokinase. Reports from two laboratories (Fiek et al., 1982; Linden et al., 1982a) have indicated that the rat liver mitochondrial pore protein may be identical with the hexokinase binding protein isolated by Felgner et al. (1979) from the same source. However, until now it had been unknown whether this suggested relationship between the two proteins might also apply to tumor cells where hexokinase is bound in markedly elevated amounts. Thus, neither the hexokinase receptor protein nor the outer membrane pore protein had been purified from mitochondria of a transformed cell line. The purification data (Table I) together with results we have obtained on DCCD inhibition of hexokinase binding and DCCD labeling of AS-30D VDAC are consistent with the hypothesis that the pore-forming protein forms at least part of the hexokinase receptor complex in hepatoma cells. As shown in Figure 3, DCCD inhibits hexokinase binding to intact mitochondria at relatively low dosages (50% inhibition at less than 2 nmol of DCCD/mg of mitochondrial protein). When intact mitochondria were labeled with [^{14}C]DCCD at 2 nmol/mg of protein, only three labeled peptides were observed in a mitochondrial extract, of which the highest molecular weight band appears to correspond to AS-30D VDAC (Figure 2). Evidence has been presented that the two lower molecular weight DCCD-labeled peptides correspond to the DCCD binding protein(s) of the mitochondrial F_1F_0 ATPase and these bands can be observed in purified preparations of F_1F_0 ATPase (Houstek et al., 1981; Glaser et al., 1981).

It is perhaps important to note that, in contrast to DCCD, which is known to interact with carboxyl groups in hydrophobic environments, the water-soluble carboxyl group modifiers EDAC and Woodward's Reagent K had no effect on hexokinase binding to the receptor complex. These results would indicate that a carboxyl group directly or indirectly essential for hexokinase binding may be located within a hydrophobic area of the membrane. VDAC was purified from DCCD-treated AS-30D mitochondria, and its pore-forming activity was extensively compared with the pore-forming activity of VDAC purified from untreated AS-30D mitochondria. No difference in channel characteristics was observed, consistent with the hypothesis that the DCCD binding site is not located within the transmembrane hydrophilic VDAC channel.

The data presented above support the conclusion that the hexokinase receptor protein and VDAC are identical in tumor mitochondria. The inhibitory effects of DCCD on the hexokinase receptor complex are also of interest in that this is one of the few reported cases where DCCD inhibits a membrane protein system that is not specifically involved in proton translocation (Solioz, 1984).

Registry No. Hexokinase, 9001-51-8; *N,N'*-dicyclohexylcarbodiimide, 538-75-0.

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Fusion and Phase Separation Monitored by Lifetime Changes of a Fluorescent Phospholipid Probe[†]

Roberta A. Parente and Barry R. Lentz*

Department of Biochemistry and Nutrition, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

ABSTRACT: The sensitivity of the fluorescence lifetime of 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine (DPHpPC) to its local concentration in lipid bilayers was used to monitor both lipid mixing and phase separation occurring during membrane vesicle fusion. Vesicles containing 2 mol % DPHpPC were mixed with a 10-fold excess of vesicles devoid of probe. Upon addition of a fusogen, mixing of bilayer lipids associated with fusion was followed as an increase in the fluorescence lifetime of DPHpPC. Ca^{2+}-induced fusion of phosphatidylserine vesicles served to test the method and was shown to have an exponential half-time of 7 s. Phase separation (between the phosphatidylserine head groups of bulk lipid and the phosphatidylcholine head groups of the probe) was monitored by DPHpPC under the same conditions used to follow lipid mixing due to fusion. Phase separation was not significant until 10 min after Ca^{2+} addition and was completely reversible by disodium ethylenediaminetetraacetate addition. Vesicle aggregation induced by Ca^{2+} addition to mixed phosphatidylserine/phosphatidylcholine vesicles did not alter the DPHpPC lifetime, indicating that close association of vesicles did not promote intervesicular exchange of the probe. In addition, we have investigated the effects of Ca^{2+} on the fluorescence properties of this probe and of the head-group-labeled fluorescent probes *N*-(4-nitro-2,1,3-benzoxadiazolyl)phosphatidylethanolamine and *N*-(lissamine Rhodamine B sulfonyl)dioleoylphosphatidylethanolamine, which are used in the fluorescence energy transfer assay of Struck et al. [Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099]. Ca^{2+} was shown to quench the fluorescence intensity of the head-group-labeled probes, while the hydrophobic environment surrounding the fluorescent moiety of DPHpPC appeared to protect it from the direct influence of such water-soluble fusogens. Overall, monitoring of DPHpPC fluorescence lifetime offered some significant advantages over current methods for detecting phospholipid reorganizations accompanying membrane fusion.

An understanding of the membrane fusion process is essential to the development of a molecular appreciation of a wide range of cellular phenomena, including exocytosis and endocytosis. The molecular events of fusion are difficult to examine in complex natural membranes; therefore, well-defined synthetic and purified native lipid vesicles are the preferred system for studying bilayer changes associated with fusion.

Two classes of fluorescent assays have been used to study vesicle fusion. One detects the mixing of internally trapped vesicle contents, and the other detects the mixing of lipid bilayer components between two vesicle populations. Both types of assays are essential for unequivocal monitoring of membrane fusion, since lipid mixing could result from phospholipid exchange and contents mixing could be confused with vesicle leakage. Several assays fall into the first of these two categories (Vanderwerf & Ullman, 1980; Ellens et al., 1985;

Kendall & MacDonald, 1982; Wilschut et al., 1980). The popular lipid mixing assay of Struck et al. (1981) makes use of changes in intensity resulting from fluorescence energy transfer between *N*-(4-nitro-2,1,3-benzoxadiazolyl) (NBD) and *N*-(lissamine Rhodamine B sulfonyl) (Rh) attached to the head-group region of phosphatidylethanolamine (PE)[1] molecules. Both NBD-PE and Rh-DOPE are placed in the same vesicle population at concentrations which allow energy transfer from NBD to Rhodamine. Fusion of these vesicles

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[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DC₁₅PC, 1,2-dipentadecanoyl-3-*sn*-phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine; DPHpPC, 1-palmitoyl-2-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-3-*sn*-phosphatidylcholine; NBD-PE, *N*-(4-nitro-2,1,3-benzoxadiazolyl)phosphatidylethanolamine; Rh-DOPE, *N*-(lissamine Rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; REV, reverse-phase evaporation vesicle(s); LMV, large, multilamellar vesicle(s); Na₂EDTA, disodium ethylenediaminetetraacetate; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TLC, thin-layer chromatography; DPH, 1,6-diphenyl-1,3,5-hexatriene.