Location of the Dicyclohexylcarbodiimide-reactive Glutamate Residue in the Bovine Heart Mitochondrial Porin*

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The mitochondrial porin or VDAC (Voltage-Dependent Anion Channel), the pore-forming structure responsible for the high permeability of the outer mitochondrial membrane, was found to be one of only three mitochondrial proteins bound by [14C]dicyclohexylcarbodiimide (DCCD) at low dosages (1.5 nmol/mg of mitochondrial porin) (De Pinto, V., Tommasino, M., Benz, R., and Palmieri, F. (1986) Biochim. Biophys. Acta 813, 230-242). Treatment of intact mitochondria with DCCD results in the inhibition of their ability to bind hexokinase (Nakashima, R. A., Mangan, P. S., Colombini, M., and Pedersen, P. L. (1986) Biochemistry 25, 1015-1021). In the present study, mitochondrial porin was purified from [14C]DCCD-labeled mitochondria. The purified labeled porin was treated with the cleavage reagent CNBr and with the endoproteases trypsin and V8 from Staphylococcus aureus aureus and blotted to polyvinylidene difluoride membrane. The recovered peptides were digested with Coomasie Blue dye, excised, and sequenced. The sequences of several labeled and unlabeled peptides were obtained and then overlapped. The region containing the [14C]DCCD radioactivity was limited to 50 amino acid residues and completely sequenced. Covalently incorporated [14C]DCCD was exclusively released at the position corresponding to glutamate 72. The DCCD-reactive residue is located in the 4th of 16 predicted transmembrane amphipathic β-strands. When the sequence surrounding the DCCD site was compared to those surrounding the DCCD-reactive residue of other membrane proteins, no homology was apparent.

Hexokinase is one of the rate-limiting enzymes of glycolysis. It has been observed in a variety of cell lines (1, 2) that the degree of glycolysis depends on the levels of hexokinase bound to mitochondria. The binding of hexokinase to mitochondria is assumed to be important in the regulation of glucose metabolism, since the bound form can utilize ATP generated inside mitochondria more efficiently and is more resistant to inhibition by glucose 6-phosphate than the soluble form (3, 4). In 1979, Felgner et al. (5) isolated the hexokinase-binding protein from the outer mitochondrial membrane. Later, this protein was demonstrated to be identical with the mitochondrial porin (6, 7). Little is known, however, so far about which domain of the porin molecule binds to hexokinase and which forces are involved in this interaction.

Dicyclohexylcarbodiimide irreversibly inhibits a number of membrane-bound proteins through covalent modification of aspartate or glutamate residues (for a review see Refs. 8 and 9). Although most proteins contain several Asp and Glu residues, the high degree of hydrophobicity of DCCD results in selective modification of only a few residues per protein. In mitochondria, low concentrations of [14C]DCCD label only three polypeptides of approximately 9, 16, and 35 kDa (10). Whereas the two smaller DCCD-binding proteins were identified as components of the F0F1 mitochondrial ATPase (10), the 35 kDa DCCD binding protein was identified as the outer membrane pore-forming protein (11). DCCD is a potent inhibitor of the transmembrane proton conductivity catalyzed by the F0F1-ATPase complex, but it does not have any influence on the pore-forming activity of porin reconstituted into planar lipid bilayers (11). Significantly, treatment of intact mitochondria with DCCD resulted in inhibition of their ability to bind hexokinase (12). Since mitochondrial porin was demonstrated to be identical with the mitochondrial hexokinase receptor (6, 7), the DCCD inhibition of the hexokinase binding to mitochondria has been attributed to modification of porin (12).

In this work, we have identified the amino acid residue of mitochondrial porin modified by DCCD, with the purpose of getting some insight into the elucidation of the porin domain involved in the interaction with hexokinase and of finding out whether the DCCD-modified sequence of porin is related to DCCD-reactive sequences of other proteins.

EXPERIMENTAL PROCEDURES

Materials—Hydroxyapatite (Bio-gel HTP) was obtained from Bio-Rad, Triton X-100, acrylamide, and N,N'-methylenebisacrylamide from Serva. Celite 545 was purchased from Roth, CAPS from Sigma, and Sephacryl SE-200 was from Pharmacia LKB Biotechnology Inc. [14C]Dicyclohexylcarbodiimide was from CEA (France), and PVDF membranes and the reagents for Edman degradation were from Applied Biosystems. Trypsin and Staphylococcus aureus aureus V8 endoprotease were obtained from Boehringer.

[14C]Dicyclohexylcarbodiimide Labeling of Mitochondria and Purification of Labeled Porin—Bovine heart mitochondria prepared by standard procedures were incubated (5 mg of protein/ml) with [14C]DCCD (1.5 nmol/mg of protein) for 16-20 h at 4 °C in 0.25 M sucrose, 1 mm EDTA, 10 mM Tris/HCl, pH 7.4 (11). The suspension was centrifuged for 20 min at 9,000 x g, and the pellet was washed twice in the same buffer. The final pellet was resuspended at a protein concentration of 50 mg/ml with the same buffer.

[14C]DCCD-labeled mitochondria (300 mg of protein) were lysed by osmotic shock; after centrifugation, the pellet, mainly composed of mitochondrial membranes, was solubilized by 3% Triton X-100.
10 mM Tris-HCl, pH 7.2, and 0.1 mM EDTA at a final concentration of 5 mg of protein/ml. After 30 min at 0 °C, the solubilization mixture was centrifuged at 40,000 × g for 15 min, and the supernatant was loaded onto a dry hydroxyapatite/celite column (45 g, ratio HTP to
celte 2:1, w/w). The elution was performed with the solubilization buffer. A volume identical with that of the solubilized protein was added from the column. It contained met porin at a high purity (15). A further purification of labeled porin from nonproteic contaminants was achieved by gel filtration upon a Sephacryl SE-200 column. The protein was first precipitated with 10% trichloroacetic acid for 1 h at 0 °C, and the pellet dissolved in 1 ml of a buffer consisting of 2% SDS, 0.1 M Tris-HCl, pH 8, 1 mM EDTA, 0.1 M dithioerythritol. This fraction was loaded onto the chromatographic column (2 × 90 cm) and eluted with the same buffer. The flow rate was 18 ml/h. Fractions containing the labeled porin were pooled and precipitated at −20 °C with acetone at a ratio of 1.5. The pellet collected by centrifugation was dissolved in 300 μl of 4% SDS, 100 mM Tris-HCl, pH 7, 12% glycerol (w/v), 10 mM dithioerythritol (Buffer A) for proteolytic cleavages.

**CNBr and Proteolytic Digestions—**The labeled protein dissolved in methanol was incubated with trypsin (protein:enzym ratio 80:1) with S. aureus V8 endoprotease (protease:protein ratio 20:1) for 2 h at 0 °C. The reactions were stopped by boiling the samples for 10 min. The peptide mixtures were then separated by SDS-polyacrylamide gel electrophoresis. CNBr cleavage of labeled porin was performed with an excess of CNBr (30 mg/ml) on porin dissolved in 70% formic acid. After a 4-h incubation, the solution was diluted 10 times with water and then freeze-dried. The dried material was dissolved in water and then freeze-dried. The dried material was dissolved in 300 μl of SDS-Polyacrylamide Gel Electrophoresis and Peptide Blotting to PVDF Membranes—SDS-polyacrylamide gel electrophoresis was performed by the discontinuous Tricine SDS-polyacrylamide gel electrophoresis system of Schägger and von Jagow (14). We used a 16.5% T, 6% C (bisacrylamide:acrylamide ratio), with 13% glycerol in the separating gel. The molecular mass markers used were the BSA-Rad Laboratories standard mass markers (92.5, 66.2, 45, 21, and 14.4 kDa) and the MW-SDS-17 kit from Sigma, containing CNBr fragments of myoglobin (17, 14.4, 8.2, 6.2, and 2.5 kDa). The electrophoresis was performed at a constant voltage of 90 V for 16 h. The upper buffer contained 1 mM thiglycolate. The gel was stained with Coomassie Brilliant Blue R-250 or transferred to PVDF membranes. In the latter case, the gel was soaked in transfer buffer (80 mM CAPS, pH 10, 10% methanol) for 10 min, and the PVDF membrane was rinsed with 100% methanol for 10 min and stored in transfer buffer. Then the gel was transferred at a constant current of 150 mA for 2 h, at room temperature. Afterwards, the membrane was washed in deionized water for 5 min and stained. Usually the membrane was stained with freshly prepared 0.1% (w/v) Coomassie Blue in 50% methanol for 5 min and destained with 50% methanol, 10% acetic acid for 10–15 min. The bands of interest were excised. Initially the membrane was incubated in a 1% solution of the antiserum against the acetylated 19 N-terminal amino acids of human porin (a kind gift of Dr. F. Thinnes, Gottingen) (dilution 1:400) and then with an anti-rabbit Ig horseradish peroxidase linked antibody (purchased from Amersham). The peroxidase reaction was performed by 20 ml of a mixture of 0.05% 4-chloro-1-naphthol, 1% methanol, 0.5% bovine serum albumin in 0.1 M NaCl, 0.01 M phosphate, pH 7.0, with the final addition of 12 μl of 30% H2O2.

**Peptide Sequencing—**Coomassie-stained, well separated peptide bands, excised from PVDF membranes, were sequenced by a Pulsed-Liquid Protein Sequencer (Applied Biosystems 477A) equipped with an on-line phenylthiohydantoin-amino acids analyzer.

**Other Methods—**[14C]DCCD labeling was detected by fluorography of Coomassie Brilliant Blue-stained gels with the aid of Kodak X-Omat films. [14C]DCCD labeling of peptides was detected as above and/or by liquid scintillation counting. Coomassie-stained gels were sliced in two alternative ways: in the former, regular, 1-mm slices were obtained; in the latter, single, separated peptides were cut. The gel slices were incubated in 30% (w/v) hydrogen peroxide at 70 °C overnight, prior to addition of the scintillation fluid. The mitochondrion was measured by the biuret method using KCN to account for turbidity due to phospholipids (15). Purified protein was measured by the Lowry method modified for the presence of detergent (16).

**Results**

DCCD modification of porin was performed in intact mitochondria. After labeling, porin was purified by a standard procedure (13). Fig. 1A shows the result of the CNBr cleavage of [14C]DCCD-labeled and purified bovine heart mitochondrial porin. Three major bands were visible on Coomassie-stained gel, corresponding to the uncleaved material and to two large peptides of apparent molecular mass of 20 kDa and 13 kDa. The large peptide (20 kDa) has been found to be blocked, and it is the N-terminal part of porin. The 13-kDa peptide showed the starting sequence: NFET after Met (17, 18). The fluorography of the same gel showed that the [14C] DCCD radioactivity was present in only two of three bands, namely the uncleaved porin and the 20-kDa peptide. This result demonstrated that the modified residue(s) were confined in the N-terminal 153 amino acids.

Further sequence data were obtained by other cleavages of labeled porin. Fig. 1B shows the result of porin cleavage by S. aureus V8 endoproteinase. The peptide pattern, detected by Coomassie Blue dye, was more complicated than that obtained by CNBr cleavage. To help in choosing the more suitable peptides for sequencing analysis, more information about the peptide pattern was collected. A duplicate of the same gel was transferred to PVDF membrane and immunostained with an antiserum against the acetylated 19 N-terminal amino acids of human porin to identify peptides containing the unsequenceable N-terminal end. Another duplicate of the same gel was sliced, and the radioactivity of each slice was determined. The trace in Fig. 1B was obtained by slicing the gel. The DCCD radioactivity was concentrated in six major peaks: while one of them contained a cluster of very close bands, it was possible to separate six labeled bands for sequence analysis. The highest Mr band, containing approximately 13% total radioactivity, was recognized by the antiserum directed toward the N terminus and, consequently, identified as uncleaved material. The next two labeled bands separated by the gel (molecular masses of approximately 27 and 26 kDa), contained, respectively, 9% and 8% of the radioactivity and had the starting sequences: F^STS and T^KPYR. The third peak of radioactive (5% of total radioactivity) corresponded to a 24-kDa peptide containing the N terminus, as indicated by the immunoreaction with the specific antiserum. Two other prominent peaks of radioactivity, corresponding to single bands on the gel, were used for extensive analysis: a peptide of approximately 15 kDa, containing 40% of the radioactivity, and a peptide of approximately 5 kDa, containing 15% of the radioactivity. Both peptides gave the same starting sequence: TTKV, corresponding to position 50 in the human sequence (17). Another radioactive peak (7–8%) corresponding to a cluster of bands was not appropriate for sequence analysis. In addition, three major bands, not showing any radioactivity, were sequenced and gave starting sequences corresponding to positions 88, 121, and 177 (Fig. 1B).

A similar analysis was performed by proteolytic digestion of labeled porin with trypsin (data not shown). With trypsin cleavage, an even more complicated peptide pattern was obtained and several peptides crowded the low Mr region. However, some of the nonradioactive peptides, with the highest molecular masses (about 17–18 kDa), were sequenced and showed the starting sequences G^SLKLT and N^LAKIK.

With all the information reported above in mind, we could circumscribe the DCCD-binding site(s) to a region corresponding to amino acids 40–88 (Fig. 2). A DCCD-binding site after residue 88 could be excluded because the V8 peptide D^QLA, the trypsin peptide G^SLKLT, and other peptides...
FIG. 1. SDS-PAGE analysis of [14C]DCCD-labeled VDAC. A, [14C]DCCD-labeled purified VDAC was cleaved by CNBr. The protein was cut in two halves: the blocked N-terminal portion (acetylated Ala', ac-A1) which was [14C]DCCD-labeled, and the C-terminal portion starting after Met 154 (N155FET). B, cleavage of [14C]DCCD-labeled purified VDAC by S. aureus V8 endoproteinase gave a much more complex pattern, which was analyzed also by immunological cross-reactivity with an antibody against the N-terminal sequence of VDAC. The central lane named Coomassie shows the V8 endoproteinase pattern obtained after Coomassie Blue staining of the SDS-polyacrylamide gel of the mixture of peptides. On the left, the lane named Ab anti N-ter shows the result of the immunological reaction of the blotted peptide mixture shown above with an antibody raised against a synthetic peptide mimicking the first 19 residues of VDAC from human B-lymphocytes. On the right of the Coomassie-stained gel is reported the corresponding radioactivity measurement obtained by counting 1-mm gel slices. The arrows indicate the position of peptides sequenced afterward and their starting sequences. The relative electrophoretic migration of the MW-SDS-17 kit from Sigma, containing CNBr fragments of myoglobin, is also shown on the extreme right.

FIG. 2. Summary of cleavage and sequencing strategy used in identifying the DCCD-labeled region of VDAC. Cleavages by CNBr, trypsin, and S. aureus V8 endoproteinase generated different sets of peptides. In the figure are reported the peptides whose N-terminal starting sequences were determined together with the V8 peptide 50–93 which was completely sequenced. The N-terminal CNBr peptide was blocked. The asterisks indicate radioactive peptides.
starting in a succeeding position were not labeled. Also, the localization of a DCCD-binding site before residue 40 could reasonably be excluded because the radioactivity detected in the V8 27-, 26-, 15-, and 5-kDa peptides, all beginning after residue 40, amounted to 72% of the starting radioactivity, and another 13% was accounted for by the uncleaved protein. In the sequence 40–93, there are 6 glutamic acid and 1 aspartic acid residue (17). We thus sequenced the whole region. Aliquots of the phenylthiohydantoin-derivatives released at each cycle of sequence were analyzed for 14C radioactivity. The sequence 40–52 was obtained from the V8 27-kDa peptide: no radioactivity was detected in those cycles. The sequence 50–93 was obtained from the V8 15- and 5-kDa peptides. The result of these analyses is shown in Fig. 3. The [14C]DCCD radioactivity was released in both cases at position 72. No significant radioactivity was found to be associated with the other cycles of the Edman degradation. In order to identify the amino acid present at position 72, the same peptides, obtained by cleaving unlabeled porin, were sequenced and showed the presence of glutamic acid at position 72. A further control was performed: after the 43rd cycle, when the sequence became illegible, the PVDF membrane containing the peptide was taken from the Sequencer cartridge and extracted with formic acid, and the radioactivity was counted. No radioactivity was detected, thus confirming that the labeled amino acid was confined before residue 93. We thus conclude that there is only 1 DCCD-modified residue in the sequence of mitochondrial porin and it is localized at glutamic acid 72.

In this work, we sequenced about two-thirds of bovine heart mitochondrial porin (data not shown). The missing parts are the blocked N terminus and the C terminus. The sequenced bovine heart porin shows about 95% identity with the human B-lymphocyte porin sequence (17). In particular, we have to point out that the bovine heart sequence 50–93 is identical with the corresponding one in human porin.

**DISCUSSION**

Of the functional groups present in a protein, DCCD can interact with carboxyls, sulfhydryls, and tyrosines but in the most commonly observed type of DCCD-protein interaction only carboxyl residues have so far been found to be implicated. When DCCD is allowed to interact with a carboxyl residue, a stable N-acylurea adduct can be formed. If it is a nearby amino group of an amino acid side chain, an inter- or an intramolecular ‘zero-length’ cross-link is formed. These reactions are common to all carboximidates. What makes DCCD a very unusual carbodiimide is its exceedingly hydrophobic nature combined with its reactivity. If a protein possesses a hydrophobic pocket that can be occupied by DCCD and if this pocket also contains a DCCD-reactive (carboxyl-) group, preferential interaction at this site takes place (8). This concept is supported by the fact that similarly small, but hydrophilic, carbodiimides such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) do not interact with sites that can be specifically labeled with DCCD, although they may react with one or several sites of a protein.

Mitochondrial porin can be labeled with a rather high specificity by DCCD. The DCCD binding to porin is very stable, and, thus, the possibility of the formation of an intramolecular zero-length cross-link should be, in our opinion, ruled out. In this work, we have located the DCCD-binding residue of mitochondrial porin at Glu72. This is the first step, from the porin side, toward the molecular understanding of the hexokinase-porin interaction.

The examination of the surrounding sequence suggests several comments. (i) The surrounding sequence does not possess a special hydrophobicity. In proteolipid (Fig. 4), the DCCD-reactive residue is located in a stretch of apolar residues comprising several Ala, Phe, and Leu residues. In porin, Glu72 is located between a Thr and a Lys, both polar residues. In a model of transmembrane arrangement we proposed previously (19), Glu72 would be localized in the 4th of 16 amphipatic β-strands which cross the membrane, namely in a region where alternating polar and apolar residues occur. Since the actual evidence and views about the three-dimensional structure of porin channel are in favor of a β-barrel monomer (20), a thin carbon backbone should surround the water-filled interior of the pore (21). This means that the hydrophobic pocket in which Glu72 residues could be formed mainly by the hydrophobic core of the phospholipid bilayer, instead of by amino acid residues. The localization of the DCCD-binding site at Glu72 is experimental evidence in favor of our proposed model of transmembrane arrangement of the porin sequence (19). In the model, the DCCD-reactive residue stands inside the membrane, while other proposed models, mainly based on the results of a site-directed mutagenesis study (22), put the corresponding region outside of the membrane.

(ii) DCCD modification of mitochondrial porin has been indicated to be responsible for the inhibition of the interaction of mitochondria with hexokinase (12). In a recent paper (23), a chimeric reporter construct, containing the 15-amino acid N-terminal domain of hexokinase coupled to the chloramphenicol acetyltransferase, was shown to be able to bind to mitochondria in a DCCD-sensitive fashion. The conclusion was that the 15-amino acid N-terminal of hexokinase is the docking domain sufficient and responsible for binding to
mitochondria. Examination of this 15-amino acid sequence shows the very hydrophobic sequence MIAAQLL-AYYFTDLK, with two charged residues, D\textsubscript{13} and K\textsubscript{15} at the end. On the other side, Glu\textsubscript{7}' is supposed, from our model of hexokinase, to be involved in the proton translocation across biological membranes. Glu\textsubscript{7}' and a corresponding positive residue present in hexokinase could be seen as sliding along the external side of the p-barrel, the ion flux would remain undisturbed. The interaction between porin and hexokinase could thus occur along a very hydrophobic, rather rigid structure: the N-terminal, docking moiety of the hexokinase could be seen as sliding along the external part of the wall separating the aqueous interior of the pore from the hydrophobic membrane moieties. The DCCD binding would produce a large steric hindrance to the interaction between the hexokinase N-terminal and the corresponding region in porin. This hypothesis could explain the result that the pore-forming activity of VDAC is not influenced at all by the DCCD modification (11): if it would occur on the external side of the \( \beta \)-barrel, the ion flux would remain undisturbed.

A problem remains to be explained: why the binding of hexokinase to mitochondria is specific, why such a hydrophobic stretch docks the protein only to mitochondria rather than to any membrane in the cell? Former studies about the interaction of hexokinase with the mitochondrial outer membranes pointed out the importance of electrostatic forces (24). It was proposed that low salt concentrations (which enhance channelling across the membrane) would produce a large steric hindrance to the interaction between the hexokinase N-terminal and the corresponding region in porin. This hypothesis could explain the result that the pore-forming activity of VDAC is not influenced at all by the DCCD modification (11): if it would occur on the external side of the \( \beta \)-barrel, the ion flux would remain undisturbed.

In our opinion, the role of electrostatic forces could be 2-fold: (a) they could confer specificity to the hexokinase-porin binding, i.e. by an interaction between the potentially negative Glu\textsubscript{7}' and a corresponding positive residue present in hexokinase like K\textsubscript{15}, whose relevance in the process has not yet been studied and (b) they could stabilize the interaction between the bulk of the enzyme structure and the surface of the outer mitochondrial membrane which should be in rather close proximity.

On the other hand, recent results showed that the human genome contains at least two genes (called HVDAC1 and HVDAC2) for homologous porins (25). They share a 75% amino acid identity. In both sequences, a stretch of 15 amino acids encompassing Glu\textsubscript{7}' is conserved. These isoforms were expressed in a yeast strain lacking the endogenous VDAC gene, and their ability to bind hexokinase was examined. The results show that HVDAC1-containing membranes could bind hexokinase, while HVDAC2-containing membranes cannot (25). If this is true, Glu\textsubscript{7}' could not be the only residue involved in the hexokinase-binding region of porin. Glu\textsubscript{7}' is not conserved in either yeast or Neurospora crassa porin (26).

(iii) DCCD is known as a classical inhibitor of FoF\textsubscript{1}-ATPase: it reacts quite specifically and covalently with the Fo of ATPase beta subunit. It was proposed that low salt concentrations (which enhance channelling across the membrane) would produce a large steric hindrance to the interaction between the potentially negative Glu\textsubscript{7}' and a corresponding positive residue present in hexokinase like K\textsubscript{15}, whose relevance in the process has not yet been studied and (b) they could stabilize the interaction between the bulk of the enzyme structure and the surface of the outer mitochondrial membrane which should be in rather close proximity.

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### REFERENCES