MINI-REVIEW

Hexokinase-Binding Properties of the Mitochondrial VDAC Protein: Inhibition by DCCD and Location of Putative DCCD-Binding Sites

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Abstract

The outer mitochondrial membrane receptor for hexokinase binding has been identified as the VDAC protein, also known as mitochondrial porin. The ability of the receptor to bind hexokinase is inhibited by pretreatment with dicyclohexylcarbodiimide (DCCD). At low concentrations, DCCD inhibits hexokinase binding by covalently labeling the VDAC protein, with no apparent effect on VDAC channel-forming activity. The stoichiometry of [^{14}C]-DCCD labeling is consistent with one to two high-affinity DCCD-binding sites per VDAC monomer. A comparison between the sequence of yeast VDAC and a conserved sequence found at DCCD-binding sites of several membrane proteins showed two sites where the yeast VDAC amino acid sequence appears to be very similar to the conserved DCCD-binding sequence. Both of these sites are located near the C-terminal end of yeast VDAC (residues 257–265 and 275–283). These results are consistent with a model in which the C-terminal end of VDAC is involved in binding to the N-terminal end of hexokinase.

Key Words: Mitochondria; hexokinase; binding; receptor; VDAC; porin; DCCD; sequence homology.

Introduction

It has been known for over 60 years that transformed cells tend to show an increased dependence upon glycolytic metabolism for energy production (Warburg *et al.*, 1924; Cori and Cori, 1925), with the most rapidly growing cancer cells obtaining up to 60% of their total energy supply from glycolysis

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(Pedersen, 1978; Nakashima *et al.*, 1984). A direct correlation has been observed between the degree of aerobic glycolysis and the levels of hexokinase bound to mitochondria in a variety of cancer cell lines (Knox *et al.*, 1970; Weinhouse, 1972; Pedersen, 1978; Bustamante *et al.*, 1981). Binding of hexokinase to a mitochondrial outer membrane receptor (Rose and Warms, 1967; Felgner *et al.*, 1979; Parry and Pedersen, 1983) would thus appear to be of some functional significance in determining the overall rate of glycolysis. Several studies have indicated that hexokinase bound to mitochondria may function more efficiently and be less subject to regulatory control than the soluble enzyme. Mitochondria-bound hexokinase is less susceptible to inhibition by glucose-6-phosphate, a potent inhibitor of soluble hexokinase (Rose and Warms, 1967; Gumaa and McLean, 1969; Colowick, 1973; Bustamante and Pedersen, 1977). In addition, it has been reported that bound hexokinase obtains preferred access to mitochondria-generated ATP, a substrate for the enzyme (Inui and Ishibashi, 1979; Kurokawa *et al.*, 1981; Arora and Pedersen, 1988).

Wilson was the first to characterize a mitochondrial outer membrane receptor protein for hexokinase binding (Felgner *et al.*, 1979). Results from three laboratories have shown that the receptor protein is identical to the outer membrane pore-forming protein (Linden *et al.*, 1982; Fiek *et al.*, 1982; Nakashima *et al.*, 1986a), also referred to as VDAC [voltage-dependent, anion-selective channel] (Colombini, 1979) or mitochondrial porin (Zalman *et al.*, 1980). This integral membrane protein of 35,000 apparent M_r forms a transmembrane channel through which low-molecular-weight, hydrophilic solutes, including ADP and ATP, diffuse across the mitochondrial outer membrane. Binding of hexokinase to the pore-forming protein would provide an obvious mechanism by which preferred access to mitochondrial ATP could be obtained. Recent experiments in Brdiczka's laboratory have suggested that the hexokinase–pore protein complex might be directly coupled to ATP transport via the adenine nucleotide translocator at regions of contact between the inner and outer mitochondrial membranes (Brdiczka *et al.*, 1985; Ohlendieck *et al.*, 1986; Denis-Pouxviel *et al.*, 1987).

We are interested in determining the molecular mechanisms by which hexokinase binding to the receptor protein affects the relative efficiency and regulatory properties of the enzyme (Rose and Warms, 1967; Bustamante and Pedersen, 1977; Pedersen, 1978; Inui and Ishibashi, 1979; Kurokawa *et al.*, 1981; Bustamante *et al.*, 1981; Nakashima *et al.*, 1986a, 1986b, 1988; Arora and Pedersen, 1988). A first step would be to identify the binding domains by which hexokinase and the receptor protein interact. An extensive literature exists indicating that the N-terminal portion of hexokinase is involved in receptor binding (Finney *et al.*, 1984; Wilson and Smith, 1985; Polakis and Wilson, 1985). This mitochondrial binding domain on hexokinase

is highly susceptible to proteolysis, and can be removed by limited treatment with chymotrypsin or other proteases (Rose and Warms, 1967; Polakis and Wilson, 1985; Nakashima et al., 1988). The resulting enzyme possesses full catalytic activity, but is no longer able to bind to mitochondria (Rose and Warms, 1967; Polakis and Wilson, 1985). The available evidence suggests that the N-terminal, mitochondrial-binding domain of hexokinase is removed by lysosomal protease activity in those tissues where hexokinase occurs primarily as a cytosolic enzyme (Rose and Warms, 1967; Yokoyama-Sato et al., 1987). In contrast with these results on hexokinase, little is known about the hexokinase binding domain on the VDAC protein. The results summarized in the present review suggest that the C-terminal end of VDAC forms at least part of the hexokinase-binding domain.

Inhibition of Mitochondrial Hexokinase Binding by DCCD

N,N'-Dicyclohexylcarbodiimide (DCCD) irreversibly inhibits a number of membrane-bound proteins through covalent modification of aspartate or glutamate residues (Cattell et al., 1971; Beattie and Villalobo, 1982; Azzi et al., 1984; Solioz, 1984). Although most proteins contain a number of Asp and Glu residues, the high degree of hydrophobicity of DCCD results in selective modification of only a few residues per protein (Svoboda et al., 1979; Azzi et al., 1984; Solioz, 1984). At low concentrations, [^{14}C]-DCCD has been reported to label three mitochondrial peptides of approximately 9,000, 16,000, and 33,000 apparent M_r (Houstek et al., 1981). The two smaller DCCD-binding proteins have been identified as components of the F_1F_0 mitochondrial ATPase (Houstek et al., 1981). Although the 33,000 M_r DCCD-binding protein was initially identified as the inner membrane phosphate transporter (Houstek et al., 1981), subsequent studies showed that it was in fact the outer membrane pore-forming protein (De Pinto et al., 1985). We therefore decided to examine the effects of DCCD on the hexokinase-binding activity of the pore protein (Nakashima et al., 1986a).

Mitochondria isolated from either normal rat liver tissue or the highly glycolytic AS-30D rat hepatoma cell line were treated with DCCD in the absence of hexokinase. Following incubation, excess DCCD was removed by washing with bovine serum albumin-containing medium. The ability of DCCD-treated mitochondria to bind solubilized tumor hexokinase was determined as described (Parry and Pedersen, 1983; Nakashima et al., 1986a, 1988). DCCD-treated mitochondria showed an irreversible inhibition of hexokinase binding activity, with a similar concentration dependence observed for liver and hepatoma mitochondria (Nakashima et al., 1986a). Fifty percent inhibition of hexokinase binding activity occurred at a DCCD

concentration of 10 micromolar (2 nmol DCCD per mg mitochondrial protein) (Nakashima *et al.*, 1986a). The VDAC protein purified from DCCD-treated mitochondria was found to be labeled with [^{14}C]-DCCD (Nakashima *et al.*, 1986a, and Fig. 1). Assays of the channel-forming activity of purified DCCD-labeled VDAC showed no change in either the specific activity for channel formation, the ion selectivity of the bilayer-incorporated channel, or the voltage-gating characteristics of the DCCD-labeled protein versus unlabeled VDAC (Nakashima *et al.*, 1986a).

At low concentrations of [^{14}C]-DCCD, only three DCCD-binding peptides were observed in AS-30D hepatoma or rat liver mitochondria, of approximately 11,000, 18,000, and 35,000 apparent M_r (Fig. 1). In order to rule out the possibility that one of the other DCCD-binding proteins was involved in hexokinase binding, submitochondrial localization studies were performed. Both of the lower-molecular-weight DCCD-binding peptides were localized in the inner mitochondrial membrane fraction (Nakashima *et al.*, 1986b), consistent with the results of Houstek *et al.* (1981). The 35,000 M_r VDAC protein was the only DCCD-binding protein found in the outer mitochondrial membrane (Nakashima *et al.*, 1986b). Since it is well established that the hexokinase receptor of hepatoma mitochondria is localized in the outer mitochondrial membrane (Parry and Pedersen, 1983), it was concluded that DCCD inhibits mitochondrial hexokinase binding through covalent modification of VDAC. Although other possibilities exist, it would be reasonable to assume that the DCCD-binding site(s) on VDAC may be located at or near the hexokinase-binding domain of VDAC. The stoichiometry of [^{14}C]-DCCD labeling of VDAC was determined by liquid scintillation counting and Lowry protein assay (Lowry *et al.*, 1951). At a concentration of DCCD (10 μM) which resulted in approximately 50% inhibition of hexokinase binding, the ratio of [^{14}C]-DCCD to VDAC monomer was approximately 0.4 mol DCCD per mol VDAC, while at concentrations of DCCD (50 μM) which produced maximal inhibition of hexokinase binding this ratio was approximately 1.3. These results are consistent with the presence of one to two high-affinity DCCD binding sites per VDAC monomer (Nakashima, 1988).

Location of Putative DCCD-Binding Sites on VDAC

In the other DCCD-binding proteins which have been characterized to date, the Asp or Glu residues which are covalently modified by DCCD occur within a highly conserved sequence of eight amino acid residues (Solioz, 1984). Comparison with the yeast VDAC protein (Mihara and Sato, 1985) showed two regions where the yeast VDAC sequence is very similar to the
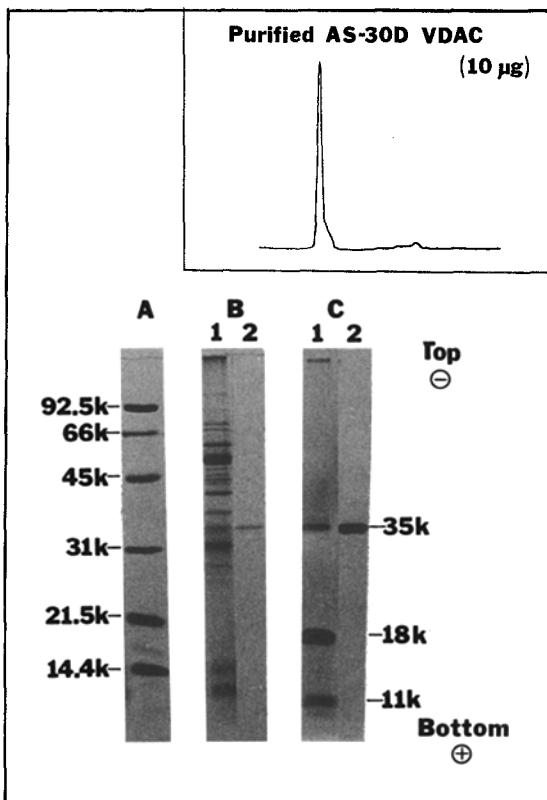
Fig. 1. Labeling of mitochondrial peptides with [^{14}C]-DCCD. Whole AS-30D mitochondria were incubated with [^{14}C]-DCCD at 2 nmol DCCD per mg of mitochondrial protein (10 μM DCCD) as described previously (Nakashima *et al.*, 1986a). The VDAC protein was purified to apparent homogeneity from the DCCD-treated mitochondria by detergent extraction and column chromatography on hydroxyapatite, DEAE-Sepharose, and CM-Sepharose (Nakashima *et al.*, 1986a). After separation by SDS-polyacrylamide gel electrophoresis, the DCCD-labeled peptides were identified by fluorography (Nakashima *et al.*, 1986a). (A) Molecular weight standards (from Bio-Rad). (B) The peptide compositions of 30 μg of whole mitochondrial extract (lane 1) and 1.5 μg of purified VDAC (lane 2) were determined by staining with Coomassie brilliant blue R-250. (C) [^{14}C]-DCCD-labeled peptides in whole mitochondrial extract (lane 1) or purified VDAC (lane 2) were identified by fluorography after treatment of the gel with En^{3}Hance (New England Nuclear). The inset to the figure shows a densitometric trace of 10 μg of purified AS-30D VDAC analyzed by SDS-PAGE and stained with Coomassie brilliant blue R-250. Reprinted with permission from Nakashima *et al.* (1986a). Copyright (1986) American Chemical Society.

Putative DCCD Binding Sites
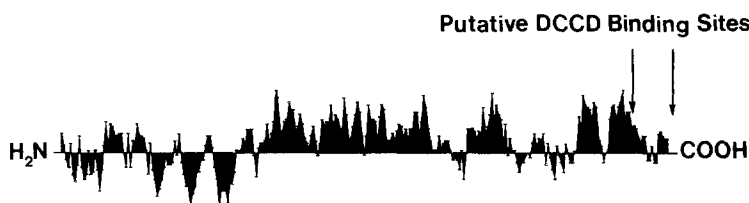
Fig. 2. Location of putative DCCD-binding sites within the sequence of yeast VDAC. The hydropathic profile of the yeast VDAC protein is shown in this figure, with the N-terminal end of the protein located on the left side of the figure and the C-terminal end on the right. [Reprinted with permission from Mihara and Sato (1985). Copyright (1985) the IRL Press Limited.] The two regions of the yeast VDAC which are homologous with the conserved DCCD binding sequence (Solioz, 1985) are indicated by the arrows.

Table I. Amino Acid Sequences of Known and Putative DCCD-Binding Sites[a]

Protein	Species	Sequence								
VDAC[b] (257–265)	Yeast	Leu	Gly	Val	Gly	Ser	Ser	Phe	Asp	Ala
(275–283)	Yeast	Leu	Gly	Trp	Ser	Leu	Ser	Phe	Asp	Ala
ATPase proteolipid	Bovine	Leu	Gly	Phe	Ala	Leu	Ser		Glu[c]	Ala
	Yeast	Leu	Gly	Phe	Ala	Phe	Val		Glu	Ala
	PS3	Ile	Gly	Val	Ala	Leu	Val		Glu	Ala
	E. coli	Ile	Val	Met	Gly	Leu	Val		Asp	Ala
Cytochrome oxidase	Bovine	Ile	Leu	Phe	Ile	Ile	Ser		Glu	Val
(subunit III)	Human	Ile	Leu	Phe	Ile	Thr	Ser		Glu	Val
	Yeast	Leu	Met	Phe	Val	Leu	Ser		Glu	Val
	N. crassa	Ile	Leu	Phe	Ile	Val	Ser		Glu	Ala

[a]From Solioz (1984).
[b]From sequence data published in Mihara and Sato (1985).
[c]Location of DCCD-binding residue.

conserved DCCD-binding sequence (Table I). The putative DCCD-binding sites on yeast VDAC occur from residues 257 to 265 and from residues 275 to 283, near the C-terminal end of VDAC. Significantly, both of these sites contain an appropriate acidic amino acid residue (Asp) which could be covalently modified by DCCD. In order to match the yeast VDAC sequence with the conserved DCCD-binding sequence it was necessary to insert a space next to the DCCD-binding residue, which is occupied by phenylalanine in the yeast VDAC sequence (Table I).

The locations of the putative DCCD-binding sites within the sequence of yeast VDAC are shown in Fig. 2. The hydropathic profile of yeast VDAC is reprinted with permission from Mihara and Sato (1985). The putative DCCD binding sites both occur near the C-terminal end of yeast VDAC, with the relevant aspartate residues located, respectively, nineteen residues and one residue away from the C-terminal end of the protein. A trans-membrane map of yeast VDAC has been constructed using the Delphi

computer algorithm of Guy (1984), assuming a β-barrel configuration of the protein (Forte *et al.*, 1987). The two putative DCCD-binding aspartate residues are located on opposite sides of the membrane, relatively close to the membrane surface in this model (Forte *et al.*, 1987). The orientations of these residues with respect to the cytosolic and intermembrane sides of the membrane are unknown. Since hexokinase binds to the outer surface of the mitochondrial outer membrane, it is likely that the hexokinase-binding domain of VDAC would be oriented toward the cytosolic side of the membrane. If the model of Forte *et al.* (1987) is correct, we would assume that only one of the two putative DCCD binding sites could be involved in hexokinase binding. This interpretation is consistent with the stoichiometry of [^{14}C]-DCCD labeling of VDAC, which suggests that occupation of one DCCD-binding site per VDAC monomer is sufficient to inhibit hexokinase binding. The number of VDAC monomers which are required to form a receptor for monomeric hexokinase is not known. Based upon the hydrodynamic properties of VDAC isolated from rat liver mitochondria, Linden and Gellerfors (1983) have suggested that VDAC forms a functional dimer within the outer membrane. Cross-linking experiments in yeast mitochondria are consistent with a one-to-one stoichiometry of bound hexokinase to monomeric VDAC (Krause *et al.*, 1986).

Although the data on putative DCCD-binding sites are consistent with the [^{14}C]-DCCD labeling studies and with the observed effects of DCCD on mitochondrial hexokinase binding, it should be noted that these experiments were not performed with yeast mitochondria. No sequence data are currently available for VDAC from either rat liver or rat hepatoma mitochondria. It is clear that DCCD does bind to VDAC from pig heart, rat liver, and rat hepatoma mitochondria (De Pinto *et al.*, 1985; Nakashima *et al.*, 1986a, and Fig. 1) and the DCCD labeling of VDAC causes inhibition of hexokinase binding (Nakashima *et al.*, 1986a). Given the presence of two regions within the sequence of yeast VDAC which are very similar to the consensus DCCD-binding sequence (Solioz, 1984), and the observed ability of rat VDAC to bind one to two moles of DCCD per VDAC monomer (Nakashima, 1988), it is considered highly likely that the mammalian VDAC protein also contains the consensus DCCD-binding sequences. Experiments are currently in progress to confirm the location of the [^{14}C]-DCCD label near the C-terminal end of rat mitochondrial VDAC and to determine the effects of site-specific mutations of yeast VDAC on its ability to bind tumor hexokinase.

Significance of DCCD-Binding Sites on VDAC

The existence of a highly conserved DCCD-binding sequence in a number of membrane proteins does not represent an adaptive response of the

organism to DCCD, since this compound does not occur in nature. Rather, it is assumed that the DCCD-binding sequence must play some important functional role related to the activities of these proteins. Although DCCD inhibits a variety of membrane proteins involved in either energy transduction or membrane transport (Cattell *et al.*, 1971; Beattie and Villalobo, 1982; Bank *et al.*, 1985; Cid *et al.*, 1987; Friedrich *et al.*, 1987; Sun *et al.*, 1987; Yagi, 1987; Beavis and Garlid, 1988; Pick and Weiss, 1988), the presence of the conserved DCCD-binding sequence has generally been considered to be specific for those proteins involved in transmembrane proton movement (Solioz, 1984). Although the pore-forming protein of the outer mitochondrial membrane will allow transmembrane proton movement to occur, the non-specific nature of transport mediated by this channel clearly distinguishes it from the previously characterized proton translocators. The question thus arises, why is the consensus DCCD-binding sequence present on the pore protein? The two obvious possibilities are (a) the outer membrane pore protein originally developed from a transmembrane proton translocator or (b) the consensus DCCD-binding sequence may be involved in functions other than H^+ transport. Aside from the putative DCCD-binding sequence, no obvious homologies exist between the yeast VDAC protein and trans-membrane proton translocators, suggesting that the first possibility is untenable. It is interesting to note that DCCD does not inhibit transport mediated by VDAC, but rather is specific for its hexokinase binding function. It is suggested that in the case of VDAC, the putative DCCD-binding sequence may be specifically involved in protein–protein binding interactions rather than transmembrane transport. An inhibition of protein–protein binding interactions by DCCD has previously been reported for the mitochondrial succinate-ubiquinone reductase complex (Xu *et al.*, 1987).

Acknowledgments

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