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The Melibiose Carrier of *Escherichia coli*: Cysteine Substitutions for Individual Residues in Helix XI

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Abstract. The melibiose carrier from *Escherichia coli* is a sugar-cation cotransport system. Previously evidence was obtained that this integral membrane protein consists of 12 transmembrane helices. Starting with the cysteine-less melibiose carrier, cysteine has been substituted individually for amino acids 374–396, which includes all of the residues in the proposed helix XI. The carriers with cysteine substitutions were studied for their transport activity and the effect of the water soluble sulfhydryl reagent p-chloromercuribenzenesulfonic acid (PCMBS). Studies were carried out on both intact cells and inside out vesicles. Cysteine substitution caused loss of transport activity in seven of the mutants (K377C, G379C, A383C, F385C, L391C, G395C and Y396C). PCMBS produced more than 50% inhibition in six of the mutants (S380C, A381C, A384C, F387C, A388C and L391C). Preincubation of the cells with melibiose protected five of these residues from the inhibitory action of PCMBS. It was concluded that the residues whose cysteine derivatives were inhibited by PCMBS probably faced the aqueous channel.

Key words: Melibiose carrier — Cotransporter — Cysteine mutagenesis — Helix XI

Introduction

The melibiose carrier of *Escherichia coli* (coded for by the melB gene) is an integral membrane protein of molecular weight 52000 [11, 20] which has 12 transmembrane helices [4, 12] with the amino- and carboxyl-termini of the protein on the cytoplasmic side of the membrane [3]. The carrier is a substrate-cation cotransport system which is unusual in its ability to use three different cations, H^+, Na^+ and Li^+. A transmembrane gradient of one of these three ions provides the energy for the accumulation of the sugar.

One approach used to study the structure and function of membrane proteins has been the use of cysteine mutagenesis. Cysteine is used to substitute for individual amino acid residues in a cysteine-less protein. The importance of the substituted residue for function can be determined. In a cysteine mutant with activity it is possible to test the effect of organic mercurials on activity. This approach has been used to study several membrane proteins: bacteriorhodopsin [5], the tar protein [9], maltoporin [6], the nicotinic acetylcholine receptor [1], the lactose carrier of *E. coli* [14] and the glucose-6-phosphate carrier of *E. coli* [19]. Recently, helix II of the melibiose carrier has been studied with cysteine mutagenesis [10]. In this study it was found that three charged residues (Arg52, Asp55 and Asp59) and the neutral residue Asn58 were essential for activity. In addition, several active cysteine mutants were inhibited by the water soluble organic mercurial (PCMBS). These data provided evidence for the location of the water channel in the carrier protein.

In the present study cysteine mutagenesis was applied to helix XI (residue 374 to residue 396) of the melibiose carrier. One reason for focusing attention on this helix was that previous studies of sugar recognition mutants [2] had shown that several of these mutations were located in helix XI. The organic mercurial PCMBS inhibited six of the active cysteine mutants and melibiose protected five of these mutants from the action of PCMBS. It was concluded that the PCMBS-sensitive residues lined an aqueous channel.

Materials and Methods

Reagents

Melibiose (6-0-α-D-galactopyranosyl-D-glucopyranoside), and PCMBS were purchased from Sigma. [^{3}H]Melibiose was a generous gift from

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Dr. Gerard Leblanc of the Department de Biologie Cellulaire et Moleculaire du CEA, Villefranche-sur-mer, France. [α-^{33}P] dATP was from Andotek. Bacteriological media were from Difco. [^{35}S]-Protein A was purchased from Amersham. All other chemicals were reagent grade. PCMBS (p-chloromercuriphenylsulfonic acid) was obtained from Sigma.

Bacterial Strains and Plasmids

E. coli DW1 (lacI$^+$ ΔlacZY ΔmelAB) [16] was used as the host strain for the plasmids when bacteria were grown for transport assays. The gene for the cysteine-less melibiose carrier was inserted into the vector, pKK223-3 (Pharmacia Biotech) as described previously [15]. This plasmid was used as the starting material for site-directed mutagenesis.

Site-Directed Mutagenesis

The Quick Change Double-Stranded, Site Directed Mutagenesis Kit (Stratagene) was used to replace the selected amino acids with cysteine. The appropriate phosphorylated mutagenic primers (ranging from 23 to 32 nucleotides long) were synthesized by Dr. Charles Dahl, Harvard Medical School.

DNA Sequencing

Double-stranded plasmid DNA was isolated with the Qiaprep Spin Miniprep Kit (Qiagen). Sequencing was done using [α-^{33} P] dATP with the Amplicycle™ Sequencing Kit (Perkin Elmer). The melB gene was sequenced from plasmid DNA using primers that anneal at approximately 200 bp intervals.

Immunodetection and Quantitation of the Melibiose Carrier

The amount of melibiose carrier present in each strain was determined as previously described by Lolkema et al. [8]. In summary, a known quantity of cells was lysed with NaOH/SDS and neutralized on nitrocellulose filters. Filters were incubated with BSA to block nonspecific binding, followed by incubation with a polyclonal antibody, anti-MBct10 [3], directed against the carboxyl-terminal ten amino acids of the protein. [^{35}S]-protein A (Amersham) was used to label the bound antibody and the amount of label was quantified by liquid scintillation counting. To correct for nonspecific adsorption, values obtained for the strain DW1/pKK223-3 (melB$^-$) were used as a background control in each experiment. Values for each mutant are presented as a percentage of wild-type protein level.

Assays for Melibiose Transport by Intact Cells

The plasmid-containing strains were grown in LB medium containing 100 μg/ml ampicillin until they reached log phase of growth. The cells were harvested and washed twice with 100 mM MOPS buffer adjusted to pH7 with Tris base and containing 10 mM NaCl and 0.5 mM $MgSO_4$ (buffer A). The cells were resuspended in the same buffer to a cell density corresponding to about 1 mg dry wt./ml. [^{3}H] melibiose (92 μCi/μmole) was added to an aliquot of the cell suspension to a final concentration of 0.1 mM. After incubation for 10 min at room temperature a 200 μl sample was filtered through a 0.65-um cellulose nitrate filter (Sartorius). The filters were immediately washed with 4 ml of buffer and counted in Liquiscint (National Diagnostics).

The effect of the sulfhydryl reagent, PCMBS, on transport was measured by preincubating the cell suspension with the indicated concentration of PCMBS for 10 min at room temperature. The radioactive melibiose was then added and processed as described. The effect of melibiose in protecting against the inhibition by PCMBS was tested by preincubating the cells with 1 mM or 10 mM melibiose for 1 min before adding the inhibitor. After 10 min incubation with inhibitor the cells (in a microfuge tube) were centrifuged, the supernatant removed and cells resuspended in radioactive melibiose. After 10 min incubation with sugar 200 μl of the sample was filtered and washed.

Assay for Melibiose Transport in Inside-out Vesicles

Forty ml of plasmid-containing bacteria were grown, harvested, washed, and resuspended in 10 ml of buffer A that contained 250 mM sucrose. Inside-out vesicles were prepared by passing the cell suspension through a French Press (SLM-Aminco) at 8000 psi [13]. Unbroken cells were removed by centrifugation and the vesicle suspension was assayed for transport. An aliquot of vesicles (321 μl) was warmed to room temperature and the PCMBS was added. The suspension was incubated for 10 min at room temperature and [^{3}H] melibiose (25 μCi/umol) was added to a final concentration of 0.1 mM. After 60 sec the vesicle suspension was filtered through a 0.22-μm nitrocellulose filter (GSTF, Millipore) and the filter was washed with buffer containing sucrose. The filter was then counted in Liquiscint in the presence of 0.1% Triton X-100.

Results

Cysteine Mutagenesis of Residues in Helix XI

Site-directed mutagenesis was applied to residues in helix XI to convert them individually to cysteine. The cysteine-less melibiose carrier [15] on a plasmid was used in these studies. In all cases, only the single desired amino acid change was found after the derivative gene's complete DNA sequence was determined.

Measurement of the Amount of Melibiose Carrier in the Membrane

When constructing mutations in membrane proteins it is always a possibility that the introduced amino acid change will disrupt proper membrane insertion and/or stability of the protein. To determine the relative amount of carrier protein present in the membrane of each mutant immunoblots were performed using a polyclonal antibody directed against the C-terminal ten amino acids of the carrier. The amount of melibiose carrier protein expressed in each strain is presented as a percentage of the wild-type value (Table 1). The expression was moderate to good in all strains except K377C which gave 10% expression and F385C which gave 5% expression.

Phenotype on Melibiose MacConkey Indicator Plates

Each of cysteine mutants in DW2 (melA$^+$ ΔB) was streaked on a melibiose MacConkey indicator plate to

Table 1. Phenotype and expression by the single replacement derivatives of the cysteine-less melibiose carrier

Cell	Phenotype on melibiose MacConkey plate[a]	Expression (% of cysteine-less parent[b])
Cysteine-less	Red	100
M374C	Red	37 ± 12
V375C	Red	56 ± 15
V376C	Red	87 ± 4
K377C	White	10 ± 5
G378C	Red	59 ± 11
G379C	White	29 ± 2
S380C	Red center	22 ± 6
A381C	Red	99 ± 20
F382C	Red	96 ± 6
A383C	White	57 ± 10
A384C	Red	60 ± 10
F385C	White	5 ± 1
F386C	Red	108 ± 7
I387C	Red	105 ± 26
A388C	Red	171 ± 27
V389C	Red	64 ± 5
V390C	Red	23 ± 4
L391C	White	64 ± 5
G392C	Red	75 ± 11
M393C	Red	126 ± 4
I394C	Red	94 ± 12
G395C	Pink center	44 ± 4
Y396C	White	40 ± 2

[a] Cell tested was DW2/plasmid.
[b] Expression was measured by the immunoblot method.

Table 2. Transport of melibiose[a] by cysteine mutants[b] in helix XI with three cations[c] (In/Out)[d]

Cell with mutation	H^+	Na^+	Li^+
Cysteine-less	16 ± 2.5	164 ± 0.5	133 ± 14
M374C	15 ± 1.5	123 ± 7.9	108 ± 1.9
V375C	3.5 ± 0.4	64 ± 7.4	15 ± 1.9
V376C	27 ± 0.6	167 ± 2.0	218 ± 0.7
K377C	1.3 ± 0.1	3.3 ± 0.8	1.3 ± 1
G378C	10 ± 0.4	88.6 ± 4.0	86.5 ± 3.9
G379C	0	0	0
S380C	10 ± 0.4	98.4 ± 2.0	85 ± 2.3
A381C	18 ± 0.3	90 ± 0.8	40 ± 2.1
F382C	20 ± 0.6	159 ± 8.4	84 ± 6.0
A383C	4.6 ± 0.6	8 ± 1.3	2.6 ± 0.3
A384C	9 ± 0.2	164 ± 12	97 ± 3.5
F385C	0	0	0
F386C	15.8 ± 0.7	146 ± 4.0	124 ± 8.0
I387C	19.4 ± 0.9	148 ± 5.4	130 ± 0
A388C	13.6 ± 0	146 ± 5.4	97 ± 0.7
V389C	15 ± 2	174 ± 20.5	136 ± 13
V390C	17.3 ± 0.4	80.4 ± 2	72 ± 1.1
L391C	10.9 ± 0.6	83.6 ± 3.4	67.8 ± 2.9
G392C	14.6 ± 0.5	143 ± 14	104 ± 4.8
M393C	10.2 ± 0.3	159 ± 4.3	117 ± 5.5
I394C	11.2 ± 0.4	125 ± 6.9	86.5 ± 1.6
G395C	18.6 ± 0.7	65.6 ± 2.0	67.8 ± 1.1
Y396C	0	0	0

[a] Melibiose concentration was 0.1 mM.
[b] Cells used were DW1/mutant plasmid.
[c] Na^+ and Li^+ were 10 mM; H^+ was from 100 mM MOPS.
[d] Melibiose concentration inside the cell/outside the cell.
The standard deviation was calculated on the basis of three independent determinations.

determine the phenotype. When DW2 containing the plasmid for the cysteine-less carrier is streaked on these plates the clones are bright red. Melibiose enters the cell, then is split by α-galactosidase into glucose and galactose. These sugars are fermented by the cell to acidic products which cause the pH indicator to give a red color. Under these conditions most of the mutants give a red color except for seven strains which give a white or pink phenotype (K377C, G379C, S380C, A383C, F385C, L391C, G395C and Y396C).

Effect of Na^+ or Li^+ on Membrane Transport of Melibiose

Membrane transport of melibiose was tested in DW1/melB plasmid strains containing different cysteine substitutions in the melB gene on the plasmid. Since DW1 is lacking the melA gene no α-galactosidase is produced and the melibiose taken up cannot be metabolized. Under these conditions the sugar accumulates in the cell to concentrations much higher than the medium. In a typical experiment cells are exposed to 0.1 mM radioactive melibiose for 10 min. Cells containing the normal melB gene exposed to melibiose in the presence of Na^+ accumulate the sugar approximately 160-fold higher than that in the external medium. In the presence of Li^+ sugar accumulation is approximately 130-fold.

Strains K377C, G379C, F385C and Y396C fail to accumulate melibiose under all conditions (Table 2). Strain A383C shows only 6% of normal activity with Na^+ while V375C and G395C show slightly less than 50% activity in the presence of Na^+. The pattern of cation stimulation of transport varied from one strain to another. V376C showed greater sugar accumulation than normal for H^+ and Li^+. On the other hand V375C and A381C showed reduced transport with Li^+. The remaining strains showed a pattern of cation stimulation similar to the normal.

The Effect of PCMBS on Melibiose Accumulation in Intact Cells

The sulfhydryl reagent, PCMBS, is a hydrophilic membrane impermeable compound and can thus be used to identify cysteines that are in a hydrophilic environment and available to the surface. Intact cells from each single-cysteine derivative strain were incubated with 100

Table 3. PCMBS[a] inhibition of melibiose transport[b] by cysteine mutants in helix XI

Cell with mutation	% activity without PCMBS	% activity with PCMBS
Cysteine-less	100	95 ± 0.5
M374C	75 ± 4.8	71 ± 4.3
V375C	39 ± 4.5	30 ± 2.8
V376C	102 ± 1.2	34 ± 0.5
K377C	2 ± 0.5	0
G378C	54 ± 2.5	58 ± 0.5
G379C	0	0
S380C	60 ± 1.2	1 ± 0.5
A381C	55 ± 0.5	1 ± 0
F382C	97 ± 5.1	8.2 ± 2.1
A383C	5 ± 0.8	0
A384C	100 ± 7.4	5 ± 0
F385C	0	0
F386C	89 ± 2.4	76 ± 2.3
I387C	90 ± 3.3	13.3 ± 1.6
A388C	89 ± 3.3	16 ± 2.7
V389C	106 ± 12.5	80 ± 5.9
V390C	49 ± 1.2	50 ± 1.6
L391C	51 ± 2.1	1 ± 0
G392C	87 ± 8.6	81 ± 6.3
M393C	97 ± 2.6	73 ± 6.7
I394C	76 ± 4.2	49 ± 4.9
G395C	40 ± 1.2	26.6 ± 4.8
Y396C	0	0

[a] PCMBS concentration was 100 μM.
[b] Melibiose concentration was 0.1 mM, Na^+ concentration was 10 mM, in 100 mM MOPS.
The standard deviation was calculated on the basis of three independent determinations.

μM PCMBS for 10 min before melibiose transport was measured. Six of the strains showed a major inhibition with PCMBS: S380C, A381C, A384C, I387C, A388C and L391C (Table 3). These experiments were repeated with inside-out vesicles (Fig. 1). The same six mutants showed strong inhibition by PCMBS. The positions of these amino acids are shown in Fig. 2.

Melibiose Protection Against the Inhibitory Effect of PCMBS

If the residue inhibited by PCMBS was close to the sugar binding site then the addition of nonradioactive melibiose prior to the inhibitor should protect the carrier from inhibition by PCMBS. The six strains that showed transport inhibition by PCMBS were tested for the possibility that preincubation of the cells with melibiose might protect the transport system against inhibition. First, the effect of different concentrations of PCMBS on transport was tested. The concentration of inhibitor that gave approximately 80% inhibition of transport was used for these experiments. In the first series of experiments cells were preincubated with 1 mM melibiose for a minute prior to addition of PCMBS. In most experiments the cells were then centrifuged, the supernatant (containing melibiose) removed and buffer containing radioactive melibiose was used to resuspend the cells. After 1 min incubation the cells were filtered, washed, and assayed for radioactive melibiose. The preincubation of cells with melibiose protected the cell against inhibition of transport by PCMBS in five cases (C380, C381, C387, C388, C391) (Table 4). In the case of inside out vesicles C384, C387, and C388 which have significant transport activity and strong inhibition by PCMBS 10 mM melibiose did not protect them against inhibition by PCMBS (50 uM). However, C391 was protected to a moderate extent against PCMBS (50 uM).

Discussion

There is extremely little information available on the three-dimensional structure of the melibiose carrier. Hydropathy plots [20], melB-phoA gene fusion experiments [4, 12] and proteolytic digestion experiments [7] provide evidence for the two-dimensional structure. The protein consists of 12 transmembrane α-helices. Little is known concerning the helices that line the aqueous channel and the region of each of these helices that faces the channel. Evidence has been presented that helix II is close to helix IV [17] and that helix IV is close to helix XI [18]. A recent publication from this laboratory [10] concerning cysteine mutagenesis of helix II indicated that one surface of the helix faced the aqueous channel. The present experiments provide evidence for one region of helix XI that faces the aqueous channel. Recently data have been presented concerning the interaction between different helices in the lactose carrier of *E. coli* [21].

In the present study each amino acid residue from position 374 to 396 was individually changed to cysteine in the cysteine-less strain. These residues constitute the transmembrane helix XI. The substitution of a cysteine residue resulted in a severe loss of transport activity in seven of the mutants: K377C, G379C, A383C, F385C, L391C, G395C and Y396C. In two cases there was extremely little carrier protein expressed in the membrane. K377C showed 10% of normal expression and F385C showed 5% normal expression. This may be due to a defect in insertion of the protein into the membrane or due to instability. In the other cases the expression was from moderate to good. In the case of L391C the cell was white on MacConkey plates but it transported moderately well in the presence of 10 mM NaCl. It is possible that the 86 mM NaCl on the MacConkey plate inhibits transport enough to produce a white clone.

Studies with PCMBS were particularly informative. This organic mercurial is highly water soluble so that it reacts with cysteine residues only if this group is in an

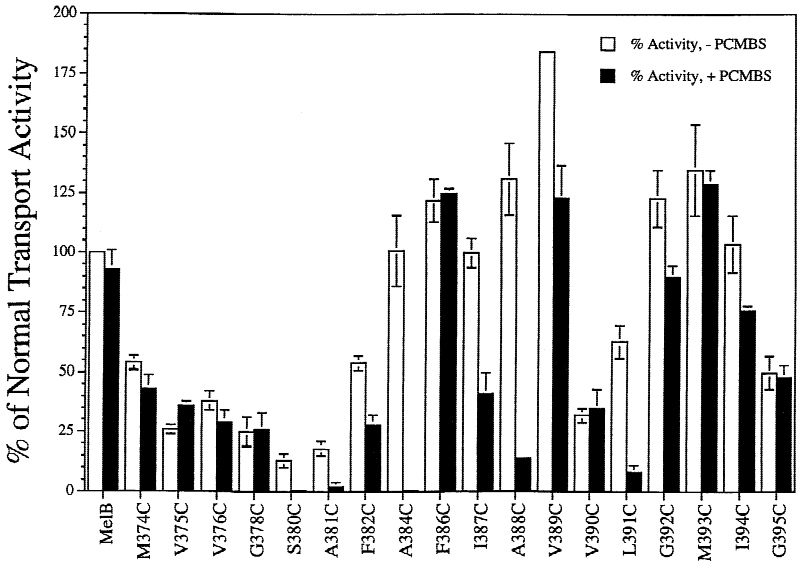

Fig. 1. The effect of PCMBS (100 uM) on melibiose transport in inside out vesicles.

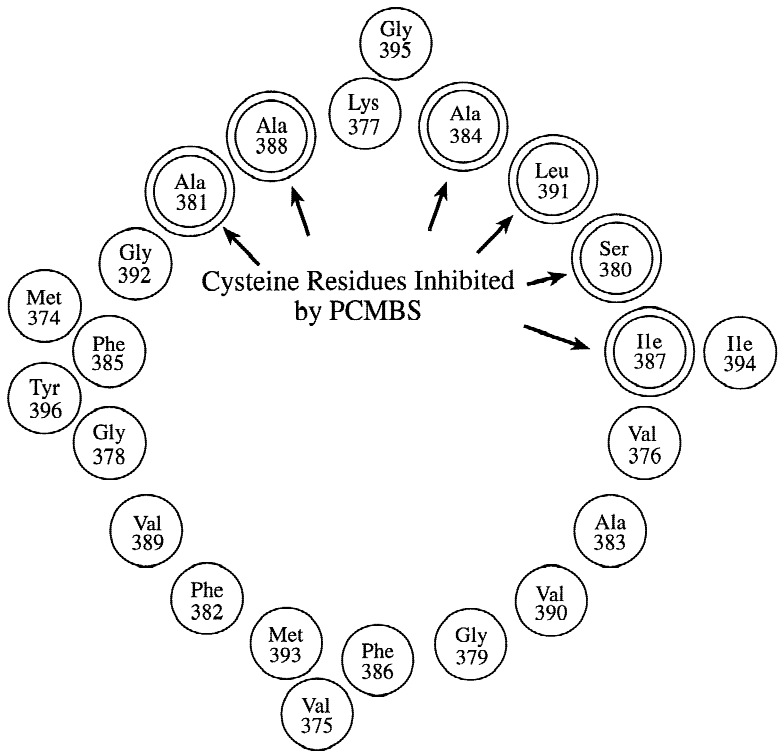

Fig. 2. Cysteine residues inhibited by PCMBS. A helical wheel plot of residues in helix XI viewed from the periplasmic surface of the membrane. Residues inhibited by PCMBS are indicated with arrows.

aqueous environment. Cysteine residues facing the phospholipids of the membrane are not susceptable to this reagent. A group of six cysteine replacements (S380C, A381C, A384C, I387C, A388C and L391C) resulted in melibiose carriers that were strongly inhibited by the hydrophilic mercurial PCMBS (Table 3). Inhibition resulted when the PCMBS was placed on either the periplasmic surface of the cell membrane (intact cells) or the cytoplasmic surface of the membrane (inside-out vesicles). There are several possible explanations of how PCMBS causes inhibition of transport. The presence of PCMBS in the melibiose channel could act as a physical barrier preventing entry of melibiose or preventing effective interaction of melibiose with its binding site. In addition, PCMBS might alter the position of the transmembrane helices in such a manner as to exclude melibiose from its normal binding site. The protection against PCMBS inhibition by preincubation of cells with

Table 4. The effect of preincubation of cells with melibiose on the inhibition of the carrier by PCMBS

Cell with indicated mutation	% Remaining transport activity	
	PCMBS	Melibiose + PCMBS
380[a]	20 ± 0.5	101 ± 8.6
381[b]	25 ± 6.5	57 ± 11
384[c]	14 ± 2.6	8 ± 0.2
387[d]	31 ± 4.5	143 ± 17
388[d]	32 ± 1.5	46 ± 2.6
391[e]	16 ± 0.5	48 ± 9

[a] pCMBS = 3 μM; Melibiose = 1 mM
[b] pCMBS = 20 μM; Melibiose = 1 mM
[c] PCMBS = 10 μM; Melibiose = 1 mM
[d] pCMBS = 100 μM; Melibiose = 1 mM
[e] pCMBS = 10 μM; Melibiose = 1 mM

melibiose indicates that five of the six residues inhibited by PCMBS may be in or near the melibiose binding site. Alternatively, the melibiose might induce a conformational change that renders one of the residues inaccessable to PCMBS.

The location of the cysteine residue in each of the six mutants that are inhibited by PCMBS gives us additional information concerning the physical position of helix XI. Figure 2 shows a helical wheel plot of residues in helix XI. The residues that are inhibited by PCMBS are found only on one face of the helix. For these residues to be available to the hydrophilic inhibitor this region of the helix must face the aqueous channel. The only charged residue in helix XI (Lys 377) is located in this region that faces the aqueous channel. In agreement with this orientation of the helix the position of several large aromatic residues F382, F385, F386 and Y396 is on the hydrophobic face of the helix. Similar studies with cysteine mutants of helix II indicate that the charged residues (R52, D55, and D59) face the aqueous channel. In unpublished experiments we have provided evidence for a salt bridge between K377 and D59. Thus there is a close physical proximity between helix XI and helix II.

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