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Multiple crystal forms of hexokinase I: new insights regarding conformational dynamics, subunit interactions, and membrane association

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Abstract Hexokinase I is comprised of homologous N- and C-terminal domains, and binds to the outer membrane of mitochondria. Reported here is the structure of a new crystal form of recombinant human hexokinase I, which complements existing crystal structures. Evidently, in some packing environments and even in the presence of glucose and glucose 6-phosphate the N-terminal domain (but not the C-terminal domain) can undergo oscillations between closed and partially opened conformations. Subunit interfaces, present in all known crystal forms of hexokinase I, promote the formation of linear chains of hexokinase I dimers. Presented is a model for membrane-associated hexokinase I, in which linear chains of hexokinase I dimers are stabilized by interactions with mitochondrial porin.

Key words: Mammalian hexokinase; Glycolysis; Conformational dynamics; Subunit interface; Protein-membrane interaction; Human

1. Introduction

Hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) catalyses the phosphorylation of glucose, using ATP as a phosphoryl donor. Four isoforms of hexokinase exist in mammalian tissue [1]. Isozymes I, II, and III have molecular weights of approximately 100 000 and exhibit 70% sequence identity [2]. The N- and C-terminal halves of isozymes I–III are similar in sequence and protein fold, a consequence perhaps of the duplication and fusion of an ancestral gene [2–5]. Mammalian hexokinase IV (glucokinase) and yeast hexokinase isoforms A and B have molecular weights of 50 000 and are similar in sequence to both halves of hexokinase I [2].

Hexokinase I is a 'pacemaker' of glycolysis in insulin independent tissues such as brain, erythrocytes and heart [6,7]. The C-half of the type I isozyme is active [8,9], whereas the N-half exhibits no activity [8,10]. Glucose 6-phosphate (Gluc-6-P), the product of the hexokinase reaction, is a potent inhibitor of the type I isozyme. Phosphate (P_i), probably in concert with glucose [11], reverses the inhibition of Gluc-6-P by binding to a site located entirely within the N-terminal half of the enzyme [11,12]. Hexokinase I in vivo is associated with pores of the outer membrane of mitochondria, specifically at tight junctions where the inner and outer membranes of the mitochondrion are in contact [13]. In its membrane associated state, hexokinase I is putatively an oligomer [14–17]. Membrane-associated hexokinase may be one component of a multiprotein complex, which channels ATP to hexokinase I and ADP back to the matrix of the mitochondrion [13,18,19].

Crystal structures of recombinant human hexokinase I complexed with either glucose/Gluc-6-P or glucose/P_i [11,20] demonstrate two conformational states for the C-terminal domain, similar to the open and closed forms of yeast hexokinase [21,22]. In the same crystal structures, however, the N-terminal domains are always closed. In the glucose/P_i complex, glucose and P_i bind to closed N-terminal domains of a hexokinase I dimer, whereas in the glucose/Gluc-6-P complex N-terminal domains of the dimer are closed with single glucose and Gluc-6-P molecules. Clearly P_i must bind to the N-terminal domain and cause a conformational change which relieves product inhibition. The most reasonable conformational change is a transition from an open to closed conformation [11].

Multiple crystal forms of a protein under the same state of ligation can often provide insight regarding the conformational dynamics of the protein, as well as reveal clear preferences for protein subunit interactions. Presented here is a second crystal form of the glucose/Gluc-6-P complex of hexokinase. Although the conformation of the dimer in the new crystal form is essentially identical to its predecessor, significant differences in lattice contacts have a profound influence on the dynamics (as measured by thermal parameters) of the N-terminal domains. In addition, one set of packing contacts between dimers are identical in the two crystal forms, and are related to a lattice contact observed in the glucose/P_i complex. That interface could facilitate efficient packing of hexokinase I dimers on a membrane surface.

2. Materials and methods

Recombinant human hexokinase I was prepared as described previously [11,20]. Crystals of hexokinase I were grown by the hanging drop method. The enzyme, stored in 25 mM potassium phosphate, pH 7.0, and 2 mM glucose, was transferred to a solution of 20 mM Gluc-6-P, pH 7.5, by repetitive cycles of concentration-filtration and redilution. 3.5 μl of the resulting solution (20 mg/ml protein concentration) was combined with an equal volume of precipitant solution containing 6–7% (w/v) polyethylene glycol 8000 (Sigma), 20 mM Gluc-6-P, 1 mM glucose, 0.1–0.2 M sodium acetate, and 0.1 M sodium citrate, pH 5.6–6.0. The drops were equilibrated against 0.7 ml of precipitant solution. Crystals grew as prismatic needles to $0.5 \times 0.2 \times 0.2$ mm.

Prior to data collection crystals were transferred to a solution of 12% (w/v) polyethylene glycol 8000, 10 mM Gluc-6-P, 5 mM glucose

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Abbreviations: Gluc-6-P, glucose-6-phosphate; P_i, phosphate

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and 0.1 M sodium citrate, pH 6.0. X-ray data were collected on SSRL beam line 7-1, using a wavelength of 1.08 Å and an 18-cm image plate detector (MAR Research, Hamburg) with a crystal-to-detector distance of 22 cm. Each of 130 frames were collected over an oscillation range of 0.9° and 15 s at a temperature of 4°C. Approximately 10 frames were collected before the onset of significant radiation damage, whereupon an unexposed portion of the crystal was translated into the X-ray beam. Two crystals were required for a complete data set. Data were processed with DENZO/SCALEPACK [23] and programs from the CCP4 suite [24].

X-ray data were consistent with the space group $P2_1$. The asymmetric unit is an entire dimer, and the solvent content of the crystal is 66% of its total volume. As evidenced by a self-rotation function, the non-crystallographic two-fold axis of the dimer is 56° from the two-fold screw axis. Molecular replacement using the previous glucose/Gluc-6-P complex [20] resulted in an unambiguous solution. Rigid body refinement of the initial molecular replacement solution gave a correlation coefficient of 0.67 and an *R*-factor of 0.39 for data to 4 Å resolution. Further refinement used XPLOR 3.851 [25] and parameters from Engh and Huber [26], as described elsewhere [20]. Model building employed a Silicon Graphics workstation (Indigo2 XL) and the software TOM [27]. Non-crystallographic restraints were applied to all protein atoms of the dimer (excluding 41 residues in lattice contacts), and resulted in better *R*-factors during refinement. Restraints were applied to thermal parameters as before [20]. Ligands were modeled against omit electron density maps based on phases from the first cycle of refinement.

Surface areas involved in lattice and subunit contacts were calculated using programs in the CCP4 suite [24].

3. Results and discussion

The new crystal form of recombinant human hexokinase I, complexed with glucose and Gluc-6-P, belongs to the space group $P2_1$ ($a = 83.8$, $b = 178.0$, $c = 88.4$, $\beta = 91.2°$) and diffracts to a resolution of 2.4 Å. Resource limitations (synchrotron time) limited data collection to 2.8 Å. The model reported from refinement has good stereochemistry and *R*-factors (Table 1). Coordinates and structure factors have been deposited with the Protein Data Bank at Brookhaven. The uncertainty in the positions of atoms is approximately 0.3 Å. On the basis of PROCHECK [28], all residues fall within the generously allowed regions of the Ramachandran plot and other categories of geometry are as good or better than those of a typical structure of 2.8 Å resolution. Thermal parameters for the model vary from 5 to 100 Å^2.

The previously reported glucose/Gluc-6-P complex [20] ($P2_1$, $a = 83.4$, $b = 121.8$, $c = 122.5$, $\beta = 92.6°$) and the crystal form reported here exhibit different packing modes of essentially the same hexokinase I dimer. Glucose and Gluc-6-P bind to each N- and C-domain, and all domains are in a closed conformation. The root-mean-square deviation over all corresponding Cα atoms in the two glucose/Gluc-6-P complexes is 0.93 Å, significantly higher than the deviation of

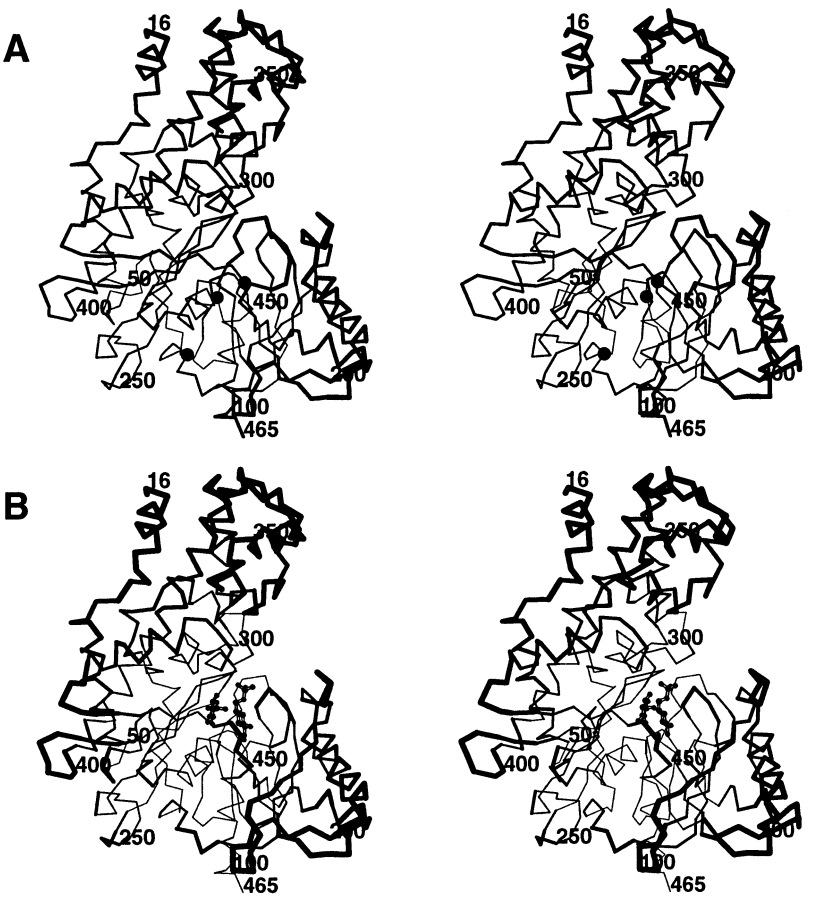

Fig. 1. Variation of thermal parameters based on a hinged oscillator model. A: Stereoview of the magnitude of observed displacement of Cα positions due to the hinged conformational change of the C-terminal domain of hexokinase I. Line thicknesses are scaled according to the magnitude of Cα displacements. Thickest lines represent displacements as large as 7.0 Å and thin lines represent displacements of 0.7 Å. Filled circles represent the centers of three hinges which allow the rigid body movements in the small and large subdomains (see [11] for details). B: Stereoview of the variation in thermal parameters of the N-terminal domain of chain A of the previous glucose/Gluc-6-P complex. Line thicknesses are scaled according to *B*-values. Thickest lines represent *B*-values above 70 Å^2 and thin lines represent *B*-values below 20 Å^2. Ball-and-stick models represent bound glucose and Gluc-6-P molecules.

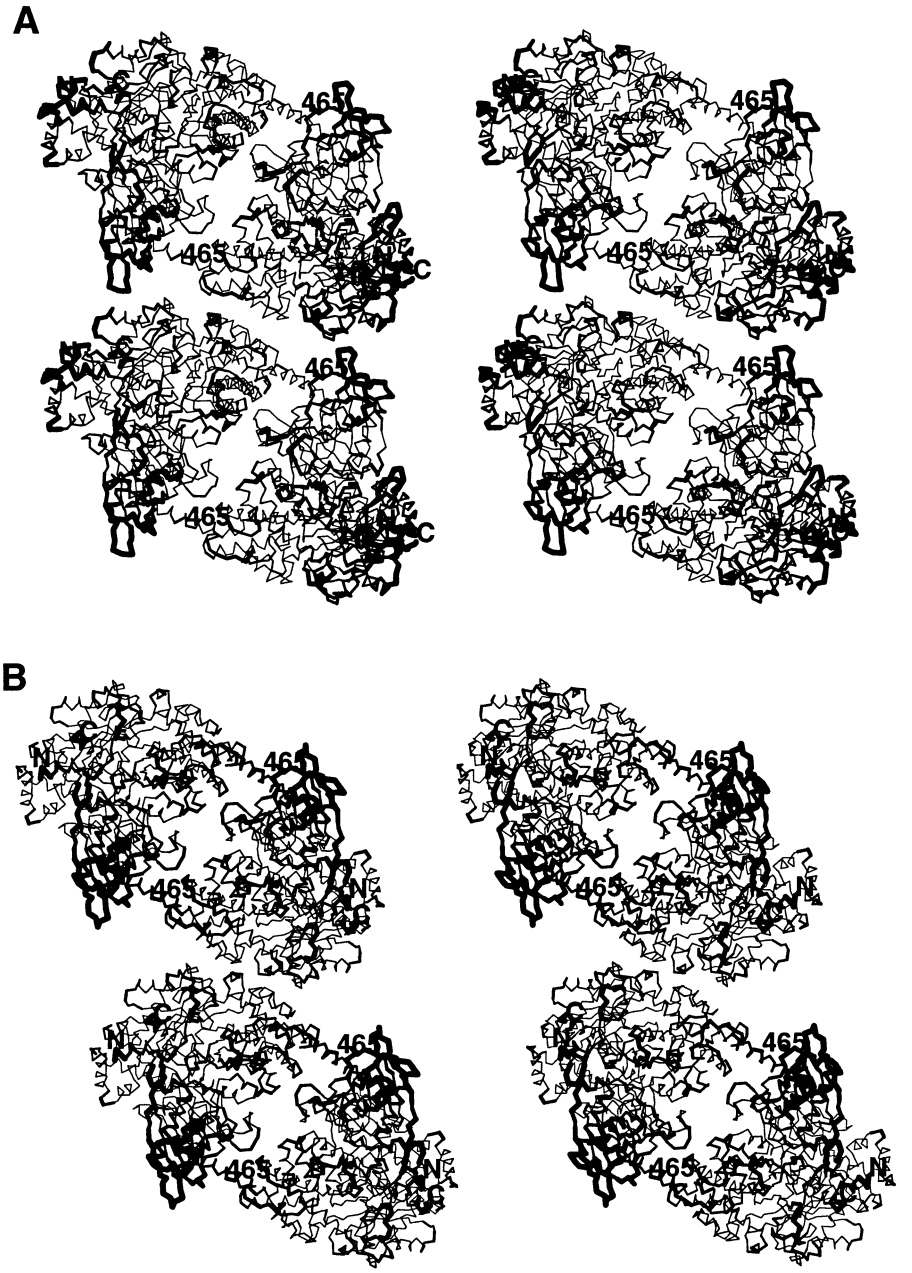

Fig. 2. Stereoview of contacts between dimers generated by the *a* interface. A: Contact observed in new glucose/Gluc-6-P complex. The identical contact exists in the previous glucose/Gluc-6-P complex [20]. B: Contact observed in the glucose/P_i complex [11]. Line thicknesses scaled according to thermal parameters as in Fig. 1.

individually superimposed domains (approximately 0.3 Å). The difference above reflects rigid body displacements of 0.2–0.5 Å for individual domains, presumably a consequence of different packing environments.

The N-terminal domains in the previous glucose/Gluc-6-P complex have uniformly high thermal parameters relative to the C-terminal domains [20]. (The average thermal parameters for the N-terminal domains of chains A and B are 58 and 46 $Å^2$, respectively, as compared to the 35 and 39 $Å^2$ of the C-terminal domains.) In the new complex, domain-averaged thermal parameters are lower and more uniform (Table 1). The *B*-values for ligands in all domains are comparable to *B*-values of nearby side chain atoms in both the previous and new structures. Furthermore, the domains all adopt the same closed conformation, as noted above. Hence, the differences in domain-averaged thermal parameters of the two crystal forms must originate from differences in the crystalline packing environment. The disorder exhibited by the N-terminal domains of the previous crystal form can arise from static packing displacements and/or the conformational dynamics of individual domains.

The elevated thermal parameters of the N-terminal domains of the previous crystal form, relative to the new form, may be due to the hinged oscillation of the N-terminal domains between a closed and partially opened conformation. Indeed, the transition between open and closed conformers of the C-terminal half is due to the hinged movement of its small and large subdomains, and a similar conformational change has

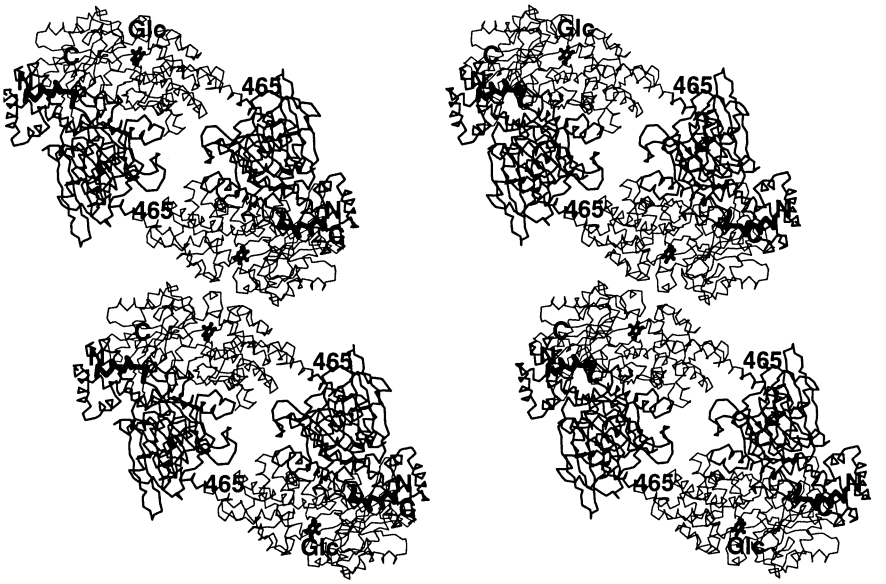

Fig. 3. Model for membrane associated dimers of hexokinase I. The N-terminal helices, drawn in boldlines, mark the location of structural elements necessary for membrane association. Glucose molecules (labeled Glc) mark the open active sites of C-terminal halves of hexokinase I. Porin molecules, with a stoichiometry of two per hexokinase I dimer, could interact with N-terminal elements and C-terminal halves of adjacent dimers.

been proposed for the N-terminal domain [11]. Hinged oscillations between closed and open domain conformations produce a characteristic distribution in the displacement of Cα atoms, which compares favorably with the observed distribution in thermal parameters of the previous crystal form (Fig. 1). Some elements of secondary structure, however, clearly behave independently of a hinged oscillation model. For instance, the extended loop 95–105 has thermal parameters well in excess of those projected by a hinged oscillation, and segment 445–450 (corresponding to a known hinge element of the C-terminal domain [11]) also departs from a hinged oscillation model. Furthermore, the analysis above cannot distinguish between static packing disorder (the presence of closely related, but fixed conformations) and dynamic disorder (free oscillation between conformational states). Nonetheless, the kind of disorder exhibited by the N-terminal domains suggests that Gluc-6-P and glucose together are only marginally effective in stabilizing a closed conformation in the previous crystal form. On the other hand, irrespective of packing environment, glucose and Gluc-6-P stabilize closed C-terminal domains, with little evidence of static and/or dynamic disorder. The above is consistent with a recent study in directed mutation and kinetics which supports a low affinity site for Gluc-6-P at the N-terminal domain and a high affinity site at the C-terminal domain [12]. In conjunction with the glucose/P_i complex, which presents an open C-terminal domain in the context of a similar hexokinase I dimer [11], the above suggests the absence of significant energy barriers to conformational transitions of individual domains in the structural context of the dimer.

Protein-protein interactions within the two glucose/Gluc-6-P crystal forms and the glucose/P_i crystal form reveal similar (and extensive) interfaces between polypeptide chains. These extensive interfaces, which define dimers of hexokinase I, each represent a larger area of contact (by 3- to 6-fold) than other subunit interfaces. Only a contact between the C-terminal halves (Fig. 2) is strictly conserved in the two glucose/Gluc-6-P complexes. This contact (hereafter called interface *a*) may represent a significant contribution to the free energy of crystallization. Crystals of both glucose/Gluc-6-P complexes grow as prismatic needles. The axis defined by the needle represents the direction of most rapid growth, and reflects the rapid concatenation of hexokinase I dimers (by way of this *a* interface) into linear chains. A modified form of the *a* interface appears in the glucose/P_i complex (Fig. 2), which also promotes the growth of prismatic needles. Furthermore, all examples of the *a* interface (which include the C-terminal domain both open and closed) involve residues with low B-values (Fig. 2), representing some of the most conformationally stabile structures of hexokinase I. Hence under conditions of crystallization, hexokinase I likes to dimerize and form extended linear chains, comprised of juxtaposed dimers.

Table 1
Statistics of data collection and refinement

Resolution limit (Å) of data	2.8
No. of measurements	169 203
No. of unique reflections	62 017
Completeness of data (%) overall/last shell	95/67 (2.9–2.8 Å)
R_{sym}[a]	0.065
Resolution range for refinement	8–2.8 Å
No. of reflections in refinement[b]	49 239
Total number of atoms	14 362
Total number of solvent sites	182
R-factor[c]/R_{free}[d]	0.188/0.247
Mean *B*-values (Å^2)	
N-terminal half (chains A/B)	35/36
C-terminal half (chains A/B)	26/33
Root-mean-squared deviations	
Bond lengths (Å)	0.007
Bond angles (deg.)	1.2

[a] $R_{sym} = \Sigma_j \Sigma_i |I_{ij} - \langle I_j \rangle| / \Sigma_i \Sigma_j I_{ij}$.
[b] Only reflections with $|F_{obs}| > \sigma(F_{obs})$ were used in the refinement.
[c] *R*-factor $= \Sigma ||F_{obs}| - |F_{calc}|| / \Sigma |F_{obs}|$, for all reflections $|F_{obs}| > 2\sigma$.
[d] *R*-factor based on 10% of the data excluded from refinement.

The crystallographic information above may be useful in modeling porin-associated hexokinase I at the outer membrane of the mitochondrion, but accurate information regarding the stoichiometry of porin and hexokinase I in such membrane complexes is essential, and for the most part unavailable. One can, however, estimate a total number of porin molecules of 42 000 in a single rat liver mitochondrion, assuming 1.5% of the total mitochondrial protein as porin [29], an M_r for porin of 30 000 and 7.2×10^9 mitochondria per milligram of mitochondrial protein [30]. Similarly, on the basis of the specific activity of purified hexokinase (60 units/mg), the units of hexokinase I that can bind per milligram of rat liver mitochondria (1.1 units/mg, [17]) and an M_r of 100 000 for the hexokinase I monomer, approximately 15 000 monomers can bind to a single mitochondrion. The ratio of porin monomers to hexokinase monomers is approximately 3. Choosing a lower limit of 0.5% for the total mitochondrial protein as porin gives a ratio of 1 porin monomer for each hexokinase I monomer.

Hexokinase I binds with cooperativity to the mitochondrial membrane (Hill coefficient of 3 [13]), and hence interactions must occur between hexokinase I subunits. As noted above, the most significant interactions observed in crystal structures of hexokinase I are the subunit interface of the dimer and the aforementioned interface *a*. These two types of interface lead to the association of hexokinase I dimers as in the glucose/P_i complex (Fig. 3). The N-terminal elements, necessary for membrane association [31], project from the same face of the assembly of hexokinase I dimers. Furthermore, the C-terminal domains from two separate dimers are in contact and face the membrane with open active sites. A porin dimer with outer orifices facing the C-terminal domains, could further stabilize this pairing of hexokinase I dimers, by interacting with N-terminal elements from adjacent hexokinase I dimers. This basic unit, if extended as a chain, would account for the binding cooperativity of hexokinase I to the membrane, and be consistent with a one-to-one ratio of porin to hexokinase I monomer. Other models are possible with different porin to hexokinase I ratios, but they share the common attribute of being linear chains of hexokinase I dimers, crosslinked by porins.

The best (and perhaps only) characterized porin complex from a eukaryotic system is from *Neurospora crassa*, where porins naturally associate into two-dimensional crystals [32]. The mitochondria of *N. crassa* are rich in porin and tight junctions, and perhaps extended porin arrays observed in such membranes are good models for porin complexes at tight junctions of other mitochondria. Hexokinase I putatively binds preferentially to the tight junctions of mitochondria [13]. The association of hexokinase I with tight junctions may be simply a cooperative assembly of hexokinase I dimers into linear chains over the surface of extended (and pre-existing) porin arrays. We caution readers, however, that the above picture is a basis for the design of experiments, rather than a rigorous account of porin-hexokinase I interactions. More precise data regarding porin-hexokinase I interactions are a prerequisite to a better model.

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