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Original Research Article

"Exclusion Zones" in Biological Cells?

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"Exclusion zones" (EZ) were observed as a lining of various depths on a hydrophilic surface in water-containing objects, including, most probably, hydrophilic membranes in biological cells. Indications for the presence of EZs inside living biological cells are scarce. A surprising property is that this kind of water moiety excludes any solutes, from emulsified objects down to molecules. One of the possible implications could be that, inside the cell, substrates for membrane-bound enzymes could have no access to their enzymes due to exclusion by this water moiety covering the hydrophilic membranes. A possible solution for this problem, created during evolution, is presented and discussed.

Keywords: Biological cells, structure-function relationships, hydrophilic membrane surfaces, membrane-bound enzymes, enzyme complexes with linkers, "Exclusion Zones", solute exclusion, accessibility of substrates to enzymes

INTRODUCTION

A very surprising property of water was discovered more than a decade ago by Gerald H. Pollack and his group, Dept. of Bioengineering at the University of Washington, Seattle. They found that hydrophilic surfaces in water-containing objects exhibit a water lining of various depths and long-range impact that excludes any solutes, in size from emulsified objects down to molecules (Zheng et al. 2006). They named this phenomenon "Exclusion Zone" (EZ).

Since then, a multitude of experiments with many kinds of approaches was performed. Their goal was and is the elucidation of the basic features of the structure and function of EZ, and the interplay of this kind of water with its environment. On the basis of the data collected so far "Pollack and his group hypothesized that the exclusion zone is composed of a liquid crystalline form of water, consisting of stacked hexagonal layers with oxygen and hydrogen in a 2:3 ratio. Ice, a solid crystalline form of water, also consists of stacked hexagonal sheets, but in the case of the ice sheets are held together by the extra protons.

Pollack proposes that EZ sheets are "out of register" - aligned so that the oxygens of each layer are frequently next to the adjacent layers. In water making up the EZs,the alignment is not perfect, but it creates more attractions than repulsions, enough to create cohesion as well as a molecular matrix tight enough to exclude even the tiniest solutes" (Charles Eisenstein in his Essay with the title "The Waters of Heterodoxy: A Review of Gerald Pollack´s Book *The Fourth Phase of Water*"). In summary: this water moiety, called by Pollack "liquid crystalline water", a fourth phase of water (Pollack 2013) besides vapor, liquid and solid, occurring at the hydrophilic surfaces in water-containing objects, appears to be responsible for the drastic effect of exclusion of all kinds of solutes. A Biologist familiar with the cellular and the ultrastructural organization of the biological cell would immediately assume that this phenomenon is unfavourable for the catalytic function of membrane-bound enzymes in the living cell.

Membranes are common structural elements in cells, and their surfaces are typical examples of hydrophilic surfaces. After all, membrane-bound enzymes – as any other kind of enzymes - has to have contact with their substrates, i.e. with solutes. According to the findings and interpretations of the Pollack group, these substrates should not be present, due to the exclusion, at the sites where the enzymes are located. In *in vitro* experiments using gels with hydrophilic surfaces, it was observed that the depth of the exclusion zones was relatively small as soon as salts were dissolved in the water. However, no data are available that would indicate that a smaller depth of the exclusion zones probably also present in biological cells (Tychinsky 2011) would be sufficient for a proper enzyme function when the enzyme is embedded into the membrane.

RESULTS AND DISCUSSION

Nature seemingly achieved the goal, i.e. accessibility of substrates to the catalytic sites of membrane- bound enzymes in biological cells, on a roundabout way: membrane-bound enzymes, i.e. the protein subunits proper of the enzymes carrying the catalytic center, are attached to the membrane *via*

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proteinaceous stalks or stems or linkers with lengths up to 15 nm; the enzyme complexes are fixed to the stalk at its top. Thus, it can be envisaged that the catalytic centers are positioned at sites sufficiently far away from the EZs, i.e. in an environment that is accessible for substrates and reaction products. From the data for the distance of the catalytic centers of the enzyme from the surface of the membrane (often in the range of 8 to 20 nm), one could perhaps get rough estimates for the depth of the EZs lining the surface of the respective membranes at the sites where the enzyme stalks are attached to the membrane.

A schematic presentation of the technical approach of a respective *in vitro* experiment using a linker is shown (Fig.1), and examples of the situations within cells are depicted (Figs.2 – 5). Further details regarding the involved organisms, enzymes and structures can be taken from the Figure Legends.

REMARK

For the future Pollack and his group expect remarkable impacts on new approaches and developments not only in various fields of technology (an example: s. Klyuzhin et al. 2008) including Biotechnology, but also in Natural Sciences in general, in Biology, in Pharmaceutics and in Medicine.

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FIGURE LEGENDS

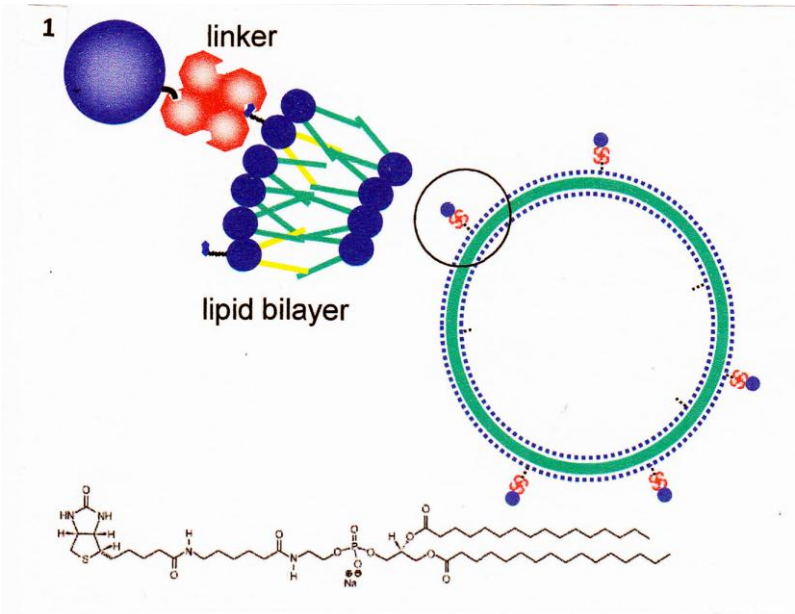

Fig.1 Assembly of enzyme-liposome complexes. At the right-hand side, a schematically drawn liposome with attached enzymes is depicted. Part of the diagram (circled) is drawn, at the left-hand side, with more details: a Streptavidin linker connects an enzyme complex with the surface of the liposome. The size of the linker determines the distance of the enzyme from the liposome. A biotin tag not occupied by a linker is also shown attached to the outside of the liposome. The linker construct makes use of the binding of biotin to Streptavidin. At the bottom of the figure a chemical formula is shown for an example of the compounds (Phosphocholines) used for the construction of the lipid bilayers of liposomes. Enzyme-liposome complexes with linkers between enzyme and liposome surface were constructed by us because we had found optimal values for the specific activities of enzymes in situations where the enzymes were not immediately in contact with the – hydrophilic – surfaces of membranes or other hydrophilic surfaces, e.g. the inner face of the monolayer of reversed micelles (Hoppert et al. 1994). Our linker constructs were used for determination of the optimal distance of a membrane-bound enzyme from the hydrophilic surface of a membrane. Our experiments were performed years before "Exclusion Zones" by Pollack´s group were discovered. - We did not have explanations for the phenomenon at that time, but we felt that such interplays of cell organization with properties of water might have been determinants for cell architecture and function during evolution (Mayer et al. 2006). (From Wichmann et al. 2003).

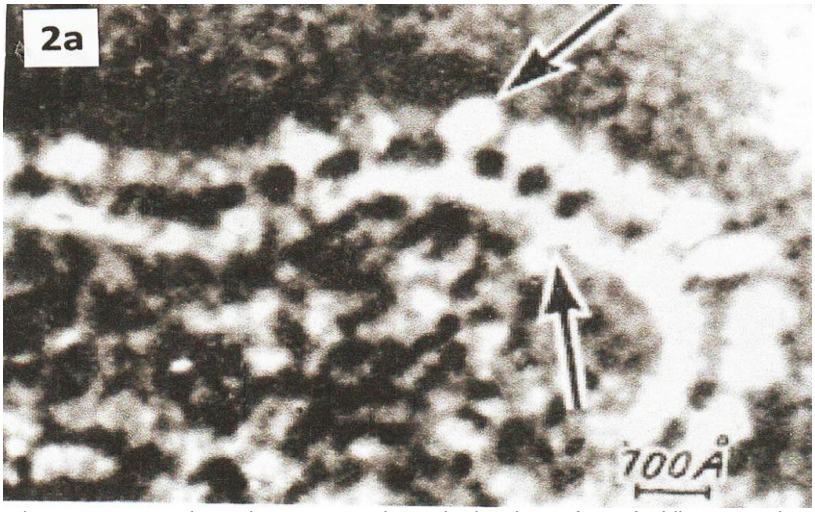

Fig.2a, Proton-translocating enzyme complexes (F1Fo ATPase) attached to the surface of a bilayer membrane (From Mayer 1986);

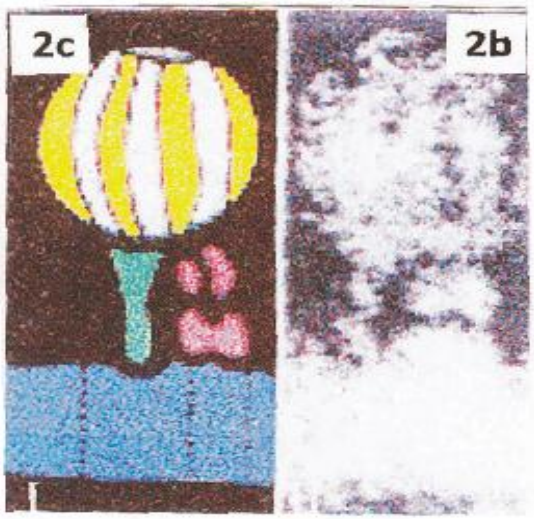

Fig.2b, electron micrograph (negative staining) of an F1Fo ATPase attached to the surface of a membrane. The head of the enzyme complex contains the subunits with the catalytic center (From Reidlinger et al. 1994); **2c,** as **2b;** drawing. (From Reidlinger et al. 1994). Both in **2b** and **2c** it is evident that not only the head, but also the stalk of the enzyme complex is composed of several protein subunits.

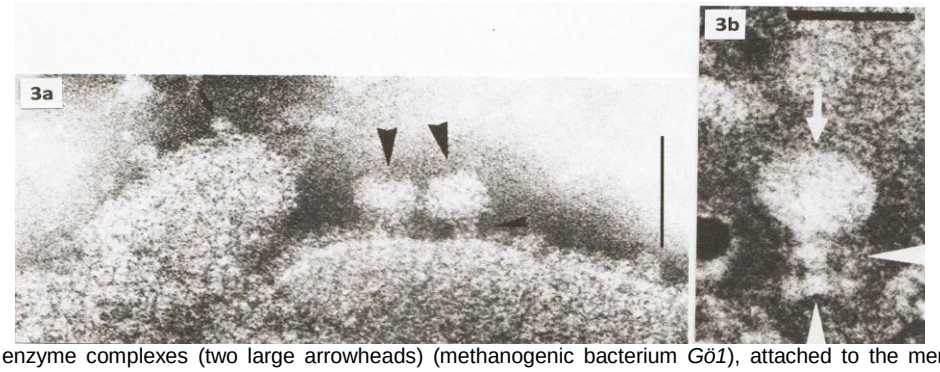

Fig.3a, Methylreductase enzyme complexes (two large arrowheads) (methanogenic bacterium *Gö1*), attached to the membrane surface (reversed orientation due to the isolation procedure) by stalks (small arrowhead). Two smaller attached enzyme complexes visible in the left half of the figure can be used for a size comparison; they are proton-translocating ATPases (s. Fig.2). Magnification bar: 100 nm b, an enzyme complex (negative staining) as depicted in Fig. 3a, shown at higher magnification and resolution. The subdivision of the complex into a head (containing the protein subunits carrying the catalytic centers), the stalk and the protein mass at the lower end of the stalk, i.e. the portion with which the stalk is inserted into the membrane, can be seen. Magnification bar: 50 nm (From Mayer et al. 1988; s. also Hoppert and Mayer 1990).

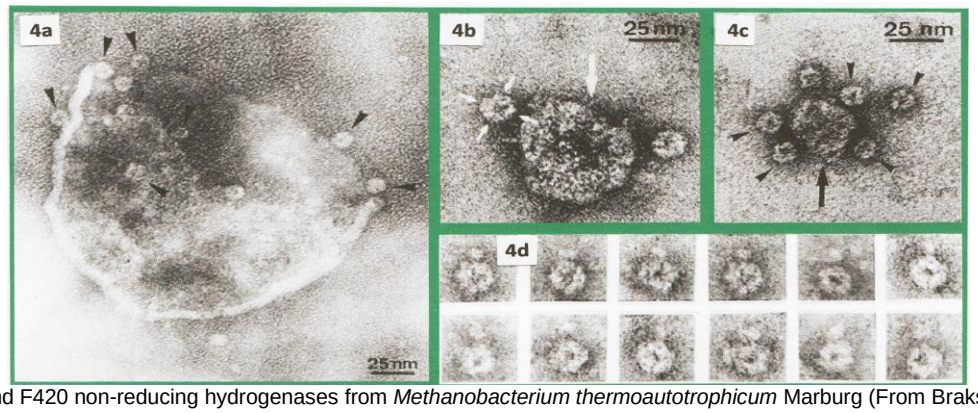

Fig.4 F420-reducing and F420 non-reducing hydrogenases from *Methanobacterium thermoautotrophicum* Marburg (From Braks et al. 1994)
4a, part of the cytoplasmic membrane after isolation; enzyme complexes (arrowheads) attached to the opened-up membrane vesicle are visible. 4b and 4c, as in 4a; the enzyme complexes are attached to the membrane surface at equal distances from the surface of the membrane. Due to the staining procedure for electron microscopy (negative staining) stalks between enzyme complexes and membrane surface cannot be detected. 4d, 12 examples of isolated enzyme complexes; due to a modified negative staining procedure, now the stalks are clearly visible.

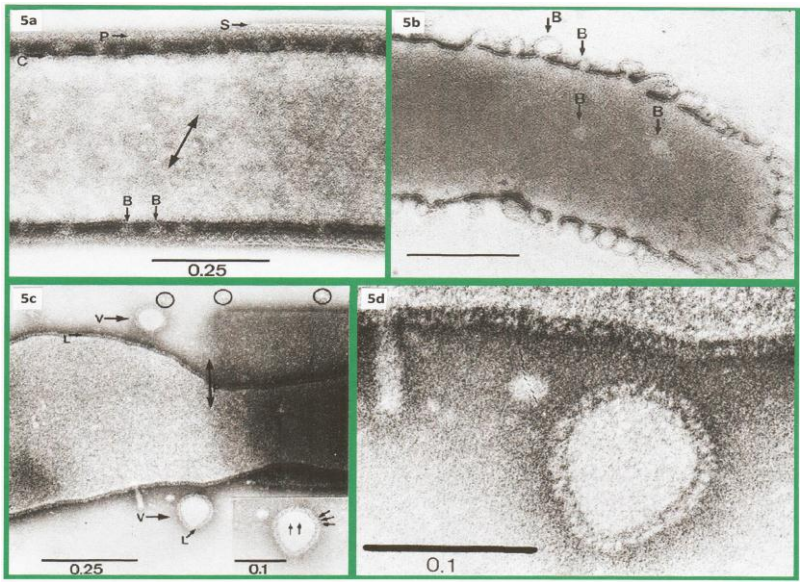

Fig.5 *Clostridium* sp. strain *EM1* during massive production of *alpha*-amylase and pullulanase. (From Antranikian et al. 1987)
5a, Electron microscopy (negative staining) of a cell in the process of loosing its surface layer (S); P Peptidoglycan layer (component of the cell wall); B, formation of blebs from the cytoplasmic membrane. 5b, as a, but a later state. The cell has completely lost its surface layer and the peptidoglycan layer. Many blebs (B) have been formed. 5c, as a and b; the cell is in the process of loosing the peptidoglycan layer (double-head arrow), and details of the organization of the blebs or vesicles (V) become visible (L, layer of amylase enzymes – group of three arrows - attached to the surface of the vesicles). 5d, as 5c, vesicle depicted at higher magnification and resolution. The amylase enzyme particles are attached to the surface of the membrane vesicle by stalks. Note that the amylase enzyme complexes are not attached to the face of a membrane pointing to the interior of the cell as in other cases (s. Figs. 2, 3 and 4), but to the outside of the membrane.