5

Metabolic Optimization by Enzyme-Enzyme and Enzyme-Cytoskeleton Associations

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1. Introduction

Probably enzymes are not dispersed in the cytoplasm, but are bound to each other and to specific cytoskeleton proteins. Associations result in substrate channeling from one enzyme to another. Multienzymatic complexes, or metabolons have been detected in glycolysis, the Krebs cycle and oxidative phosphorylation. Also, some glycolytic enzymes interact with mitochondria. Metabolons may associate with actin or tubulin, gaining stability. Metabolons resist inhibition mediated by the accumulation of compatible solutes observed during the stress response. Compatible solutes protect membranes and proteins against stress. However, when stress is over, compatible solutes inhibit growth, probably due to the high viscosity they promote. Viscosity inhibits protein movements. Enzymes that undergo large conformational changes during catalysis are more sensitive to viscosity. Enzyme association seems to protect the more sensitive enzymes from viscosity-mediated inhibition. The association-mediated protection of the enzymes in a given metabolic pathway would constitute a new property of metabolons: that is, to enhance survival during stress. It is proposed that resistance to inhibition is due to elimination of non-productive conformations in highly motile enzymes.

2. Metabolons: Enzyme complexes that channel substrates

The cytoplasm should not be regarded as a liquid phase containing a large number of soluble enzymes and particles. Instead, it has become evident that there is a high degree of organization where different lipid and protein structures associate among themselves and with other molecules. The high molecule concentration found in the cytoplasm promotes macromolecule associations such as protein-protein, protein-membrane, protein-nucleic acid, protein-polysaccharide and thus is a control factor for all biological processes (Srere & Ovadi, 1990). Indeed, the classical studies by Green (Green *et al.,* 1965), Clegg (Clegg, 1964)

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and Fulton (Fulton, 1982) have suggested that enzymes are not dispersed in the cytoplasm. Instead, enzymes are localized at specific sites where they are associated between them and with the cytoskeleton. The cytoskeleton is a trabecular network of fibrous proteins that micro-compartmentalizes the cytoplasm (Porter *et al.*, 1983). Associated enzymes channel substrates from one to another preventing their diffusion to the aqueous phase (Gaertner *et al.*, 1978; Minton & Wilf, 1981; Ovadi *et al.*, 1996).

In a multienzyme complex, intermediaries can be channeled more than once from the active site of an enzyme to the next to obtain the final product (Al-Habori, 2000; Robinson *et al.*, 1987). Channeling requires stable interactions of the multienzymatic metabolons (Al-Habori, 2000; Cascante *et al.*, 1994; Ovadi & Srere, 1996; Ovadi & Saks, 2004; Srere & Ovadi, 1990; Srere, 1987). The metabolon stability is facilitated by the compartmentalization of the cell in different organelles and structures (Jorgensen *et al.*, 2005).

There are many advantages inherent to metabolons (Jorgensen *et al.*, 2005) (Fig. 1): I) Improved catalytic efficiency of the enzymes. This is obtained by channeling an intermediary from the active site of an enzyme directly to the active site of the next. II) Channeling optimizes kinetic constants. III) Labile or toxic intermediates are retained within the metabolon. IV) Inhibitors are excluded from the active site of enzymes. V) Control and coordination of the enzymes in a given pathway is enhanced. VI) Finally, alternative metabolons may favor different pathways (Fig. 1). Most metabolons seem to be transient, opening the possibility for a quick change in some elements that allows them to redirect metabolism (Jorgensen *et al.*, 2005).

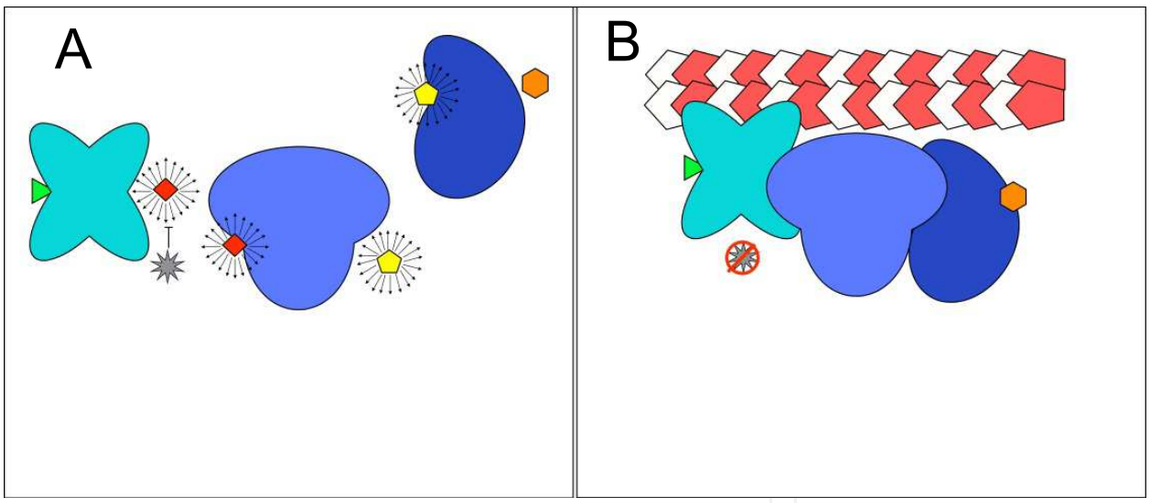

Fig. 1. Advantages of Metabolons. (A) In isolated enzymes the substrate (green), intermediaries (red and yellow) and product (orange) diffuse into the aqueous phase (little arrows). Toxic intermediaries and inhibitors (grey) are free to exit/enter the active site in each enzyme. (B) In metabolons (we show filamentous actin in red and white) channeling allows transfer of the substrate (green) from the active site of an enzyme direct to the next to obtain a final product (orange) without diffusion to the cytoplasm of intermediaries (not-depicted) are prevented, while inhibitors (grey) are excluded from the active sites.

The enzymes from the Krebs cycle are attached to the mitochondrial membrane in an enzymatic complex; this was the first "metabolon" described (Srere, 1987). In oxidative

phosphorylation, multiprotein complexes seem to associate in supercomplexes and eventually in respiratory chains resulting in controlled electron channeling and proton-pumping stoichiometry (Guerrero-Castillo *et al.*, 2011). It has been proposed that these supercomplexes constitute an exquisite mechanism to regulate the yield of ATP (Guerrero-Castillo *et al.*, 2009; 2011; Schägger *et al.*, 2001). In addition, in some organisms such as trypanosomes, glycolytic enzymes are contained in small organelles called glycosomes, where channeling is highly efficient (Aman *et al.*, 1985). Tumor cells also produce aggregates containing glycolytic enzymes (Coe & Greenhouse, 1973). Interactions between organelles such as the endoplasmic reticulum and mitochondria have been described (Dorn & Scorrano, 2010; Kornmann *et al.*, 2009; Lebiedzinska *et al.*, 2009). Mitochondria are both, the main source of ATP and inducers of cellular death (Anesti & Scorrano, 2006). Mitochondrial functions are regulated by interactions with other organelles and cytoplasmic proteins (Kostal & Arriaga, 2011). Cytoskeletal proteins such as actin and tubulin, direct mitochondria to specific sites in the cell (Senning & Marcus, 2010) and control coupling of phosphorylation by interacting with mitochondrial porin (Xu *et al.*, 2001; Lemasters & Holmuhamedov, 2006; Rostovtseva *et al.*, 2008; Rostovtseva *et al.*, 2004; Xu *et al.*, 2001). In addition to cytoeskeletal proteins, hexokinase, a glycolytic enzyme binds mitochondria in mammalians (Pastorino & Hoek, 2008), yeast and plants (Balasubramanian *et al.*, 2008) regulatin the energy yield of mitochondria as well as the induction of programmed cell death (Kroemer *et al.*, 2005; Pastorino & Hoek, 2008; Xie & Wilson, 1988). All the above data suggest that enzymes are highly organized (Clegg & Jackson, 1989) and the cytoskeleton plays an important role (Minaschek *et al.*, 1992; Keleti *et al.*, 1989; Porter *et al.*, 1983).

3. The cytoskeleton: A scaffold where metabolons are bound

The eukaryotic cytoplasm is supported by the cytoskeleton, a network of structural proteins that shapes the cell and has binding sites for different enzymes. Such sites have been identified in filamentous actin (F-actin), in microtubules and in the cytoplasmic domain of the erythrocyte band 3, which is also an anion exchanger. Glycolytic enzyme binding to actin usually results in stimulation, whereas binding to microtubules or to band 3 inhibits activity (Real-Hohn *et al.*, 2010). Actin is involved in a variety of cell functions that include contractility, cytokinesis, maintenance of cell shape, cell locomotion and organelle localization. In addition, glycolytic enzymes and F-actin co-localize in muscle cells, probably reflecting compartmentation of the glycolytic pathway (Waingeh *et al.*, 2006).

Actin is highly conserved in eukaryotic cells. It may be found as a monomer (G-actin) or as a polymeric filament (F-actin) that is interconverted in an extremely dynamic, highly controlled process. The polar actin monomers polymerize head-to-tail to yield a polar filament. Actin filaments are constituted by 8 nm diameter, double-helical structures formed by assemblies of monomeric actin with a barbed end (or plus end) and a pointed end (or minus end). The spontaneous polymerization of actin monomers occurs in three phases: nucleation, elongation and maintenance. Nucleation consists in the formation of a dimer, followed by the addition of a third monomer to yield a trimer; this process is very slow. Further monomer addition becomes thermodynamically favorable and the filament elongates rapidly: much faster at the plus end than at the minus end. In the maintenance phase, there is no net filament growth and the concentration of ATP-G-actin is kept stationary (Fig. 2).

Upon incorporation to a filament, G-actin-bound ATP is hydrolyzed. ADP and Pi remain non-covalently bound. Then Pi is released slowly. Thus, the elongating filaments contain: the barbed end, rich in ATP-actin, the center, rich in ADP-Pi-actin and the pointed end containing ADP-actin. Many actin-binding proteins regulate actin polymerization. Profilin is an actin monomer-binding protein; Arp 2/3 complex are nucleation proteins; CapZ and gelsolin regulate the length of the actin filament and the cofilin/ADP family cuts F-actin and accelerates depolymerization (Kustermans *et al.*, 2008). However, protein functions may vary; in *Dictyostelium,* CapZ prevents filament elongation and increases the concentration of unpolymerized actin; in contrast, in yeast this same protein prevents depolymerization increasing F-actin concentration (Welch *et al.*, 1997). The cytoskeleton can be rapidly remodeled by the small RhoGTPases (Rho, Rac and Cdc42), which act in response to extracellular stimuli (Kustermans *et al.*, 2008). There are exogenous natural compounds that can disturb actin dynamics (Kustermans *et al.*, 2008).

4. The glycolytic metabolon

The association of enzymes with the cytoskeleton probably stabilizes metabolons. In this regard, glycolytic enzymes such as fructose 1,6-bisphosphate aldolase (aldolase), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), piruvate kinase (PK), glucose phosphate isomerase (GPI), and lactate dehydrogenase (LDH) associate with actin. Other glycolytic enzymes such as triose phosphate isomerase and phosphoglycerate mutase bind indirectly through interactions with other enzymes. Enzyme-enzyme-actin complexes are called piggy-back interactions. Also, aldolase and GAPDH compete for binding sites (Knull & Walsh, 1992; Waingeh *et al.*, 2006).

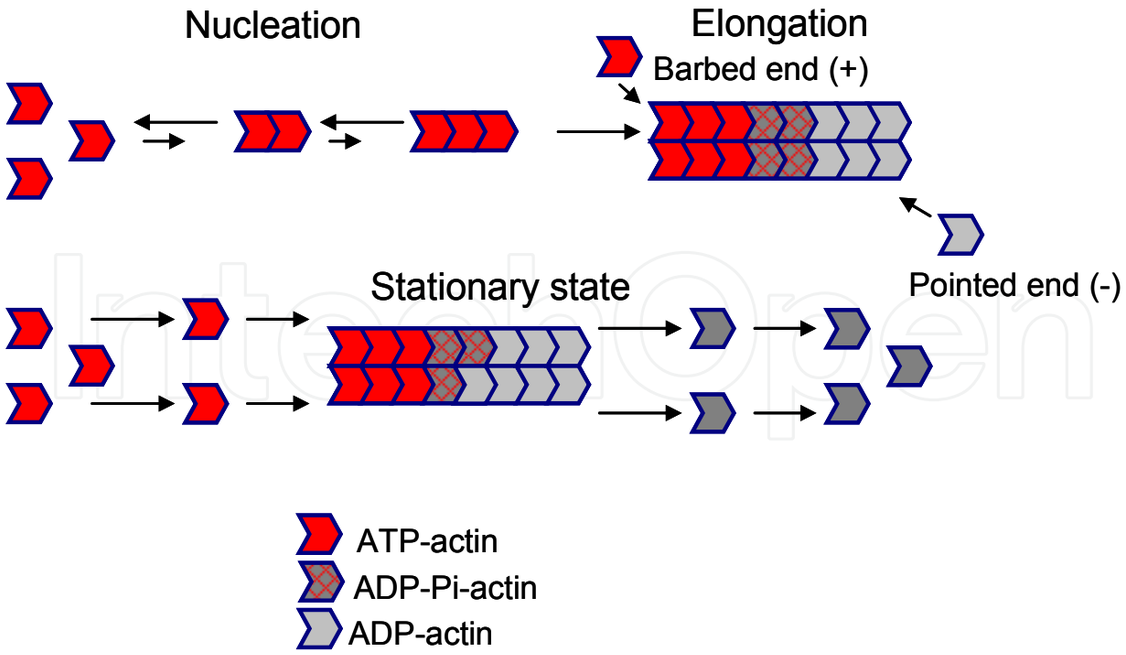

Fig. 2. **Actin polymerization.** During nucleation, actin monomers aggregate to form a trimer. Then during elongation actin filaments grow actively at both ends. Growth stops in the maintenance phase, also known as stationary phase. (Modified from Kustermans *et al.*, 2008)

Enzyme/actin interaction is regulated by ionic strength (Waingeh *et al.*, 2006). In homogenates of muscle tissue suspended in isosmotic sucrose, proteins such as F-actin, myosin, troponin and tropomyosin associate with glycolytic enzymes (Brooks & Storey, 1991). Glycolytic enzyme association to actin is not accepted universally, for instance, the F-actin/glycolytic enzyme interaction has been modeled mathematically at physiological ionic strength and protein concentrations. The results suggest that under cellular conditions only a small percentage of TPI, GAPDH, PGK and LDH would be associated with F-actin (Brooks & Storey, 1991).

Protein dynamics seem important for their interactions. Brownian dynamics (BD) simulations detect that rabbit F-actin has different binding modes/affinities for aldolase and GAPDH (Forlemu *et al.*, 2006). Some metabolites such as ATP and ADP modulate enzyme interactions and the resulting substrate channeling (Forlemu *et al.*, 2006).

A barely explored effect of the association of enzymes with the cytoskeleton is the modulation of the dynamics of actin polymerization. Such an effect has been reported for aldolase (Chiquete-Felix *et al.*, 2009; Schindler *et al.*, 2001). An interesting finding is that some growth factors, such as PGF and EGF enhance the GAPDH/cytoskeleton interaction, possibly increasing keratinocyte migration (Tochio *et al.*, 2010). Indeed, GAPDH seems to participate in cytoskeleton dynamics processes such as endocytosis, membrane fusion, vesicular transport and nuclear tRNA transport (Cueille *et al.*, 2007).

In red blood cell membranes, GAPDH, aldolase and PFK interact with an acidic sequence at the amino-terminal extreme of band 3 with high affinity (Campanella *et al.*, 2005). Under physiological conditions, the binding of glycolytic enzymes to band 3 results in inhibition of the glycolytic flux (Real-Hohn *et al.*, 2010).

Association to microtubules regulates the energetic metabolism (Keleti *et al.*, 1989; Keller *et al.*, 2007; Walsh *et al.*, 1989) at the level of some glycolytic enzymes such as pyruvate kinase, phosphofructokinase (Kovács *et al.*, 2003) and enolase (Keller *et al.*, 2007). When the glycolytic enzymes are associated and anchored to the sarcomere, ATP is produced more efficiently (Keller *et al.*, 2007). The interaction of enzymes with themselves and with the cytoskeleton confers more stability to the enzyme activity and to the whole network (Keleti *et al.*, 1989; Volker *et al.*, 1995; Walsh *et al.*, 1989). F-actin stabilizes some glycolytic enzymes of muscle and sperm (Walsh & Knull, 1988; Ovadi & Saks, 2004). That is the case of the phosphofructokinase (PFK) and aldolase where the dilution-mediated inactivation of PFK is stopped upon aldolase addition. If PFK is associated with microtubules, it still loses activity when diluted, however, in these conditions it recovers the lost activity upon aldolase addition (Raïs *et al.*, 2000; Vértessy *et al.*, 1997). All this evidence supports the existence of a cytoskeleton-bound glycolytic metabolon.

5. Compatible solutes protect cellular structures during stress

Compatible solutes are defined as molecules that reach high concentrations in the cell without interfering with metabolic functions (Brown & Simpson, 1972). These are mostly amino acids and amino acid derivatives, polyols, sugars and methylamines. Compatible solutes are typically small and harbor chemical groups that interact with protein surfaces. Indeed, some authors have proposed to call them "chemical or pharmacological chaperones" as they stabilize native structures (Loo & Clarke, 2007; Romisch, 2004). Some compatible solutes are:

glycine betaine, a thermoprotectant in *B. subtilis* (Chen & Murata, 2011; Holtmann & Bremer, 2004). Ectoine, that in halophile microorganisms confers resistance to salt and temperature stress (Pastor *et al.*, 2010). Glycerol is accumulated in yeast under high osmotic pressure (Blomberg, 2000). Glycerol stabilizes thermolabile enzymes preventing their inactivation (Zancan & Sola-Penna, 2005). The disaccharide trehalose protects against environmental injuries (heat, cold, desiccation, and anoxia) and nutritional limitations (Argüelles, 2000; Crowe *et al.*, 1984) in bacteria, yeast, fungi, plants and invertebrates. In biotechnology, trehalose is one of the best protein stabilizing known (Jain & Roy, 2008; Sampedro *et al.*, 2001).

6. Effect of compatible solutes on the activity of enzymes

Compatible solute synthesis and accumulation is triggered by harsh conditions and results in protein stabilization and enhanced survival. Proteins may be unfolded, partially unfolded or native (Chilson & Chilson, 2003). In the absence of stress, high compatible solute concentrations inhibit cellular growth, metabolism and division (Wera *et al.*, 1999), e.g. a trehalase-deficient mutant of *S. cerevisiae* subjected to heat or saline stress accumulated high amounts of trehalose and survived. However, when these mutants were returned to normal conditions they are unable to grow or sustain metabolic activity (Garre & Matallana, 2009; Wera *et al.*, 1999).

6.1 Inhibition of isolated enzymes; possible role of viscosity

Under stress, high compatible solutes change the physicochemical properties of the cytoplasm. However, the effect of the high viscosity generated by molar concentrations of compatible solutes on enzyme activity has drawn little attention. Trehalose and other polyols protect proteins from thermal unfolding via indirect interactions (Liu *et al.*, 2010). Therefore the stabilizing mechanism must rely in the modified physicochemical properties of aqueous media.

Large-scale conformational changes in proteins involve the physical displacement of associated solvent molecules and solutes. The resistance to the movement or displacement of solvent molecules is a frictional process. Kramers theory provides the mathematical basis to understand and analyze reactions at high viscosity (Kramers, 1940). The application of Kramer´s theory to proteins indicates that the movements involved in folding or in enzyme-substrate association and processing must be highly sensitive to viscosity (Jacob and Schmid, 1999; Jacob *et al.*, 1999; Sampedro and Uribe, 2004).

Studies on cellular viscosity in yeast cytoplasm showed a value of 2 cP at 30°C (Williams *et al.*, 1997). Also, in vitro determinations for 0.6 M trehalose solutions showed a viscosity of 1.5 cP at 30°C (Table 1). Therefore, one may infer that yeast cytoplasm viscosity with 0.6 M trehalose should be in the vicinity of 2.5-3 cP.

The plasma membrane H^+-ATPase from yeast depends on large domain motion for catalysis (Kulbrandt, 2004), was inhibited at all trehalose concentrations tested (Sampedro *et al.*, 2002). The rate constant for the ATPase reaction ($V_{max} = k_{cat}[E_t]$) was inversely dependent on solution viscosity; as higher the viscosity lower the reaction rate of catalysis (Sampedro *et al.*, 2002). Notably, when temperature was raised inhibition disappeared, in agreement with the fact that viscosity decreases when temperature increases (Table 1). Similar results have been obtained with Na^+/K^+-ATPase and Na^+-ATPase in the presence of polyethylene glycol and

glycerol (Esmann *et al.*, 2008). In glucose oxidase, activity inhibition by varying concentrations of trehalose was due to the promotion of a highly compact state, which correlated with the increased viscosity of the medium (Paz-Alfaro *et al.*, 2009).

TREHALOSE (M)	**0.2**	**0.4**	**0.5**	**0.6**	**0.8**
TEMP (°C)	**VISCOSITY (cP)**				
20	1.35	1.59	1.81	2.04	2.58
25	1.20	1.37	1.51	1.74	2.20
30	1.08	1.18	1.33	1.50	1.91
35	0.94	1.03	1.18	1.31	1.67
40	0.86	0.94	1.04	1.13	1.49
45	0.75	0.81	0.90	1.04	1.31

Data modified from Sampedro *et al.*, 2002.

Table 1. Viscosity of trehalose solutions at different concentrations and temperatures.

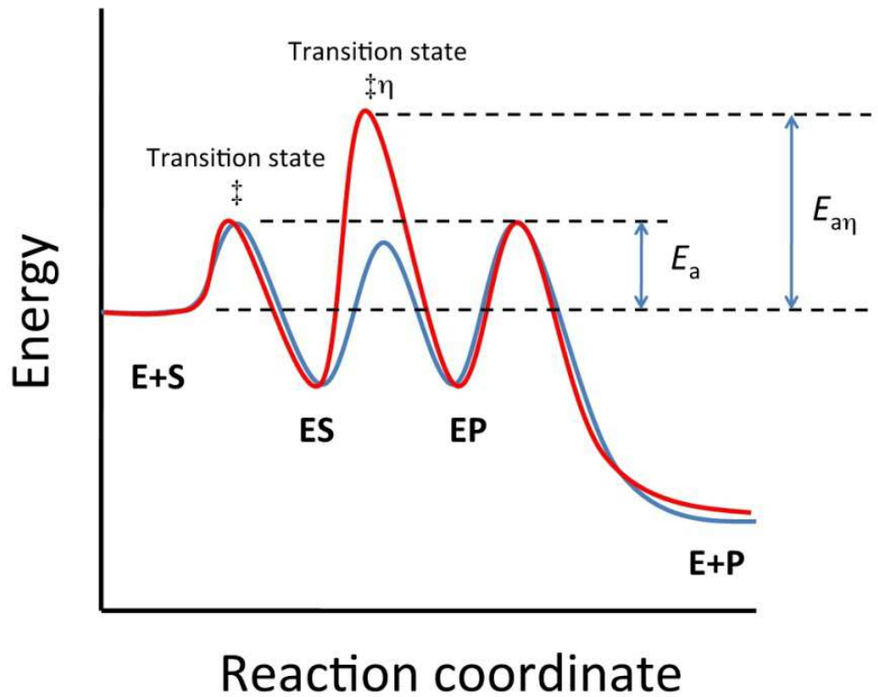

Fig. 3. Reaction coordinate diagram, comparing an enzyme reaction at normal viscosity (blue) and at high viscosity (h; red). When a diffusive protein domain process is present in the catalytic cycle, it becomes rate limiting when viscosity is high. Therefore the overall activation energy (E_a) increases.

Many enzymes are inhibited by viscosity. Glutathione reductase is inhibited at 25°C, by trehalose (70% inhibition at 1.5 M trehalose) although inhibition disappears at 40°C (Sebollela *et al.*, 2004). Also pyrophosphatase and glucose 6-phosphate dehydrogenase show temperature dependence of trehalose-mediated inhibition (Sebollela *et al.*, 2004).

Aminoglycoside nucleotidyltransferase 2''-I is inhibited by glycerol in a temperature-dependent way (Gates & Northrop, 1988). The hyaluronan-synthase from *Streptococcus equisimilis*is is inhibited by of PEG, ethylene glycol, glycerol or sucrose (Tlapak-Simmons *et al.,* 2004). At high viscosities (greater than 4 mPa s-1) different carbohydrates inhibit egg-white lysozyme (Lamy *et al.,* 1990; Monkos, 1997).

Detailed studies on diffusive protein-structural components demonstrated that for β-lactam synthase a conformational change is rate-limiting on k_{cat}. Therefore, the rate for catalysis shows a high inhibition by medium viscosity (Raber *et al.*, 2009). Crystallographic analysis of adhesion kinase-1 shows a large conformational motion of the activation loop upon ATP binding. This is an essential step during catalysis and explains the viscosity inhibitory effect (Schneck *et al.,* 2010). In the plasma membrane H^+-ATPase, the enzyme fluctuates between two structural conformations (E1↔E2) during catalysis. The N-domain (nucleotide binding) rotates 73° towards the phosphorylation site to deliver ATP to the phosphorylation site (Kuhlbrandt, 2004). In all cases, the rate-limiting step is a conformational change that seems to be the one inhibited by viscosity (Fig. 3).

6.2 Enzyme association results in protection against inhibition

Compatible solute-mediated inhibition does not seem to uniformly affect all enzymes. Furthermore, in the face of both the stress condition and the compatible solute, catabolic pathways seem to resist inhibition, thus providing the energy needed for survival (Hoffmann & Holzhütter, 2009; Hounsa *et al.*, 1998). In our hands, in a yeast cytoplasmic extract, compatible solutes inhibit the whole glycolytic pathway much less than many of its individual, isolated enzymes (Araiza-Olivera *et al.*, 2010). In contrast, anabolism seems to be shot both during the stress situation and later (Attfield, 1987). Inhibition of anabolism would explain the inability of cells to reproduce (Wera *et al.,* 1999). The mechanism for resistance to inhibition, exhibited by the catabolic enzymes is a matter of study (Marcondes *et al.,* 2011; Raïs *et al.*, 2000).

The effect of a compatible solute (trehalose) on the activity of some yeast glycolytic enzymes such as GAPDH, HXK, ALD and PGK has been analyzed. These enzymes were tested individually or in mixtures (Araiza-Olivera *et al.*, 2010). When isolated, GAPDH and HXK were inhibited by trehalose while others, such as ALD and PGK were resistant. Probably GAPDH and HXK are more motile than ALD and PGK. Remarkably, when the sensitive enzymes were mixed with the resistant enzymes a protection effect was observed. This led to analyze the whole glycolytic pathway and again, inhibition was minimal in comparison with the individual, isolated enzymes (Araiza-Olivera *et al.*, 2010). Thus, it was decided to explore the possible mechanisms underlying this effect, i.e, why some metabolic pathways, such as glycolysis resist the viscosity-mediated inhibition promoted by compatible solutes, even if they contain several viscosity-sensitive enzymes.

The protection effect was specific for each protein couple, as GAPDH was not protected by neither HXK, albumin or lactate-dehydrogenase. Also, the pentose pathway enzyme glucose 6-phosphate dehydrogenase (G6PDH) was not protected by ALD against inhibition by trehalose. Once in the complexes, probably the more flexible enzymes that are more sensitive to viscosity (Sampedro & Uribe 2004) are stabilized by the more resistant, more rigid enzymes forming a less motile, more resistant complex.

The proposal that enzyme association favors a more stable folded state would require the motile enzymes to eliminate some non-productive conformations (Villali & Kern, 2010). These associations are probably further stabilized by some elements of the cytoskeleton, such as tubulin (Raïs *et al.*, 2000; Walsh *et al.*, 1989) or F-actin (Minaschek *et al.*, 1992; Waingeh *et al.*, 2006). Thus, it is proposed that another function of enzyme association into metabolons, in addition to substrate channeling and metabolic control might be to resist compatible solute-mediated inhibition.

7. Concluding remarks

Under stress, compatible solutes accumulate to very high levels in the cytoplasm. This results in enhanced viscosity. As revised in section 6.1, viscosity is known to inhibit diverse enzymes. Indeed, high viscosity may be the mechanism by which diverse cell functions are inhibited in the presence of high compatible solute concentrations, e.g. cells are unable to. In contrast, catabolism remains active even in the presence of compatible solutes. One possible mechanism for this resistance to inhibition is probably the specific association of glyolytic enzymes among themselves and probably with the cytoskeleton. Resistance to viscosity-mediated inhibition is proposed as a novel, important property of enzyme association into metabolons. The mechanism of protection that association confers against viscosity still has to be defined. Protection of activity is needed for survival during stress.

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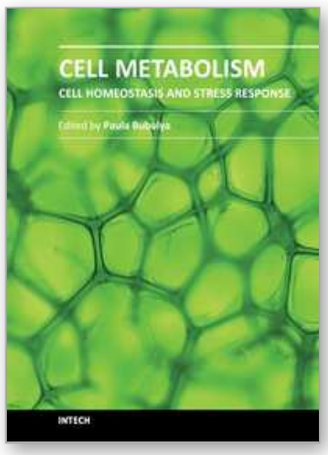

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A global research community of scientists is teasing out the biochemical mechanisms that regulate normal cellular physiology in a variety of organisms. Much of current research aims to understand the network of molecular reactions that regulate cellular homeostasis, and to learn what allows cells to sense stress and activate appropriate biochemical responses. Advanced molecular tools and state-of-the-art imaging techniques discussed in this book continue to provide novel insights into how environmental changes impact organisms, as well as to develop therapeutic interventions for correcting aberrant pathways in human disease.

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