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REVIEW

Cooperativity in regulation of membrane protein function: phenomenological analysis of the effects of pH and phospholipids

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Abstract

Interaction between membrane proteins and ligands plays a key role in governing a wide spectrum of cellular processes. These interactions can provide a cooperative-type regulation of protein function. A wide variety of proteins, including enzymes, channels, transporters, and receptors, displays cooperative behavior in their interactions with ligands. Moreover, the ligands involved encompass a vast diversity and include specific molecules or ions that bind to specific binding sites. In this review, our particular focus is on the interaction between integral membrane proteins and ligands that can present multiple "binding sites", such as protons or membrane phospholipids. The study of the interaction that protons or lipids have with membrane proteins often presents challenges for classical mechanistic modeling approaches. In this regard, we show that, like Hill's pioneering work on hemoglobin regulation, phenomenological modeling constitutes a powerful tool for capturing essential features of these systems.

Keywords Cooperativity · Phenomenological models · Protons · Phospholipids · Aquaporins · P-ATPases

Historical perspective on cooperativity studies

The cooperative regulation of protein activity is usually identified when the biological activity shows a sigmoidal pattern, instead of a hyperbolic one, when increasing ligand concentration. A pioneer mathematical modeling approach describing this sigmoidal response was proposed by Archibald V. Hill in 1910. This was a theoretical exercise to mathematically support the work of his older colleagues at the Physiological Laboratory at Trinity College, Cambridge, where Hill had obtained a studentship the previous year (Katz 1978). In 1922, A.V. Hill was awarded the Nobel Prize in Physiology or Medicine *for his discovery relating to the production of heat in the muscle*, which was his main research area. However, one of his most influential contributions was this short note on the binding of oxygen to human hemoglobin (Hill 1910).

Some years before, Bohr and colleagues showed that the hemoglobin-oxygen binding curve has a sigmoidal shape (Bohr et al. 1904). At that moment, hemoglobin was considered to be a monomeric molecule with one oxygen binding site, and the sigmoidal shape was not regarded as indicative of cooperativity since this concept was not yet elaborated as we understand it nowadays. Moreover, it was assumed that a monomeric protein binding a unique ligand molecule could not generate a sigmoidal binding curve. So, Hill found a way to mathematically describe the sigmoidal curve of oxygen binding to human hemoglobin by considering the aggregation of hemoglobin molecules when the oxygen partial pressure increased. Hill proposed that hemoglobin monomers aggregate in groups of n units, and that this complex bound n molecules of

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oxygen simultaneously. This proposal gives the famous mathematical expression known as "Hill equation":

$$Y_s = 100 \cdot \frac{K \cdot x^n}{1 + K \cdot x^n} \quad (1)$$

This equation has two variables, x (the ligand concentration, in this case the O_2 partial pressure) and Y_s (the saturation of binding sites) and two parameters, n (an aggregation coefficient that indicates the number of hemoglobin molecules aggregated), and K (the overall association constant giving account of the affinity between hemoglobin and its ligand). Interestingly, in this foundational work for cooperative regulation of proteins, there is no mention to cooperativity at all. The quaternary structure of hemoglobin was solved years later by Max Perutz using X-ray diffraction, demonstrating its tetrameric nature and disclosing that four oxygen binding sites per molecule are always present (Perutz 1963). In between those years, Gilbert S. Adair and colleagues, by applying the mass action law to the corresponding association equilibriums, derived an equation that describes the binding density as a function of the concentration of free ligands (Adair et al. 1925). This equation takes the form of a quotient of polynomials, with the degree of the polynomials corresponding to the number of binding sites. The equation includes the macroscopic equilibrium constants, one for each sequential binding step, as parameters. It provides a model-independent description of the binding isotherm which includes all possible microscopic binding models regardless of whether the binding sites are identical or different and whether interactions occur between them. Within this framework, the Hill equation would represent a limit situation where strong interactions occur among the binding sites, and partially saturated states either do not exist or do not contribute to the binding curve. This extreme condition is now referred to as infinite interaction.

A "rediscovery of the Hill work" was made by Jeffries Wyman many decades later, when introduced the Hill plot, based on the Hill equation. Through seminal works (Wyman 1964; Wyman and Gill 1990), Wyman proposed that the parameter n (thereafter called the Hill coefficient, n_H) is "closely related to the average free energy of interaction among the binding sites." This insight offered a significant breakthrough, as Wyman found that the Hill coefficient could be a practical and straightforward method, via the Hill plot, to assess cooperativity in a binding curve without the need of using the more rigorous procedure of determining the individual binding constants and calculating the intrinsic constants (Weber 1992; Cattoni et al. 2015). Another view on the meaning of Wyman's Hill coefficient is its association with fluctuations in the extent of binding, as discussed by Terrell Hill (Hill 1985) and Holt and Ackers (Holt and Ackers 2009).

The equation proposed by A.V. Hill in 1910 is still considered the primary approximation to analyze a cooperative process unless the two main hypotheses of the original work, protein aggregation and infinite interaction, have been proven to be wrong. The Hill equation that is now commonly used, and which derives from the historical one (Eq. 1), is:

$$S = F_{(S,\bar{v})} \cdot \frac{n \cdot K^{n_H} \cdot [L]^{n_H}}{1 + K^{n_H} \cdot [L]^{n_H}} \quad (2)$$

where S is the measured signal, $F_{(S,\bar{v})}$ is a function connecting the measured signal with the binding density, $[L]$ is the ligand concentration, and K represents the inverse of the ligand concentration producing half-maximal saturation on the measured signal.

Nowadays, it is commonly accepted that the Hill coefficient provides a criterion to determine the type of interaction. Indeed, the Hill equation is the most used mathematical expression to describe cooperativity in the scientific literature. It is usually accepted that: (i) if the value of the Hill coefficient is 1 (and the Hill equation becomes a rectangular hyperbola), there is no interaction, suggesting identical and independent binding sites; (ii) if Hill coefficient takes values higher than 1, the system shows "positive cooperativity," suggesting an increase in the affinity of a binding site due to the previous binding of a ligand to another site; and (iii) if Hill coefficient takes values lower than 1, the system show "negative cooperativity," suggesting that the binding of the first ligand diminishes the probability of binding for a second molecule. So, the Hill equation has been shown to be an excellent tool to understand protein–ligand cooperative interaction despite the Hill coefficient n_H and K no longer represent the physical parameters originally intended.

Another way to describe a sigmoidal curve is the so-called differential logistic function (Reed and Berkson 1928):

$$\frac{dS}{d[L]} = \frac{c}{S_{max} - S_{min}} \cdot \left(S - S_{min}\right) \cdot \left(S_{max} - S\right) \quad (3)$$

Interestingly, this function does not refer to any specific mechanism, but its rationale is that the sensitivity to the change of a given property S ($dS/d[L]$) is proportional to how far the system is from two limit situations (the asymptotes S_{max} and S_{min}). In a binding process, these asymptotes could represent the signal values corresponding to the protein without ligand bound and the full saturated ligand–protein complex. Parameters in this equation were the above-mentioned asymptotes and a coefficient c which is related to the maximal slope of the curve (occurring at the inflection point) and gives a measure of the degree of cooperativity in the process. When integrating Eq. 3 along the ligand coordinate, it is useful to group some terms in a new parameter denoted as $k_{0.5}$, which represents the ligand concentration

producing half-maximal effect. It is easy to see that it has the same meaning as the inverse of the parameter K in the Hill equation (Eq. 2). Both Eqs. (2) and (3) have been widely used to give account of sigmoidal curves providing a phenomenological view of the process under study.

Biological function of proteins almost always begins with a binding event, but often goes beyond ligand binding since the progress of the reaction (in the case of enzymes) or transport events (in case of channels) usually involves conformational changes that optimize the active site or the pore structure. In this regard, two separated events (at least) can be recognized in a cooperative response in most proteins: ligand binding and transduction of conformational information. These two steps have been identified in mechanistic models proposed to explain cooperative phenomena, such as the concerted MWC model (Monod et al. 1965), and the sequential KNF model (Koshland et al. 1966), which are limiting cases of a more general scheme as was posited by Manfred Eigen in his Nobel Lecture (Eigen 1968). The influence of these models in biochemistry has been significant, given their effectiveness in explaining experimental data. Nonetheless, they pose certain challenges, including the treatment of a fixed number of binding sites, assumptions regarding conformational changes, and the consideration of homogeneous populations of protein isoforms. It is important to acknowledge that these model assumptions may not be applicable to numerous biologically relevant systems, especially in the case of integral membrane proteins regulated by phospholipids or protons, as they exhibit intricate behaviors that require alternative modeling approaches.

Lipids and protons as cooperative ligands

There is an extensive list of ligand types that show cooperative regulation of protein function. Many examples of particularly physiological importance have been widely discussed in the literature. The most relevant, considering its historic impact and its importance in cell physiology, is the oxygen binding to hemoglobin, but also acetylcholine, and some other molecules of this kind largely reviewed as cooperative ligands. Contrasting with this, phospholipids and protons are less recognized as ligands that mediate cooperativity in the biological function of many proteins, and the interaction of these ligands with proteins is usually difficult to investigate due to the existence of multiple "binding sites" and their ubiquitous nature.

It is known that cellular membranes play a key role in modulating the structure and function of integral membrane proteins (Phillips et al. 2009; Levental and Lyman 2023). Interactions between these proteins and membrane lipids can be site-specific (structural lipids), as observed on several high-resolution structures of membrane proteins (Sun and Gennis 2019; Niu et al. 2020), or non-specific, interacting with the transmembrane hydrophobic surface without specific sites (Corradi et al. 2019). Both types are critical for consolidating the native structure and allowing the proper function of these proteins. Furthermore, non-specific interactions generate a shell of lipids surrounding the protein (formerly named annulus) which have restricted mobility and can be dynamically exchanged with the bulk lipids. On the other hand, the cooperative regulation of proteins by protons is a frequent event, but unlike classical ligands with a clear binding site, pH changes allow the protonation of several residues that can act as sensors and effectors of conformational changes which can impact in biological activity. It is worth noting that in the case of pH effects, the sigmoidal shape of the binding curve (characteristic of positive cooperativity) must be observed in a curve representing the biological effect as a function of $[H^+]$, because being pH a logarithmic function an apparent sigmoidicity appears for all types of binding (Cattoni et al. 2015).

Some cases have been selected to illustrate the variety of integral membrane proteins in which cooperative regulation by lipids or protons has been studied and the techniques employed (Table 1).

Despite the essential roles that lipids and protons play in the regulation of biological function of integral membrane proteins, mechanistic understanding of the process is sometimes limited due to different reasons such as incomplete structural and/or dynamic data or scarcity of quantitative information about the effects of these ligands on each specific binding site. For those cases, phenomenological models constitute a powerful tool for the description of experimental systems.

Phenomenological models to understand cooperative regulation

For those biological cases of cooperative regulation with limited mechanistic or dynamic information, it is still possible to analyze the cooperative response using phenomenological models, alone or combined with other biophysical tools such as molecular dynamics simulations. What do we refer to by "phenomenological models"? Despite both in biological sciences and in philosophy of science phenomenological models have been defined in different ways, all considerations are somehow related. It is not our intention to review all the literature on this topic but to use the notion of phenomenological model most frequently used in our area of research, so we follow Roman Frigg who points out that, traditionally, phenomenological models are considered as models that represent observable properties without postulating hidden mechanisms (Frigg and Hartmann 2020). Two cases of study that illustrate how a phenomenological

Table 1 Selected examples of cooperative regulation of integral membrane protein function by lipids or protons

Class	Protein	Ligand	Experimental approach	Reference
Channel	Influenza M2 protein	Proton	NMR	(Liao et al. 2015)
	PIEZO1	Proton	Patch clamp	(Bae et al. 2015)
	TASK-3 K	Proton	Patch clamp	(González et al. 2013)
	KcsA	Proton	Stopped flow fluorescence quenching	(Chakrapani et al. 2007; Posson et al. 2013; Rusinova et al. 2014)
	ASIC	Proton	Patch clamp	(Wang and Bianchi 2009)
	AQP0	Proton	Oocyte permeability assays	(Németh-Cahalan et al. 2013)
	AQPZ	PL	Proteoliposome permeability assay	(Tan and Torres 2021)
	BK	Cho	Patch clamp	(Chang et al. 1995; Lange and Steck 2016)
	GIRK2	PL	Patch clamp	(Wang et al. 2014)
Transporter	NHE-1	Proton	$^{22}Na^{+}$ uptake	(Lacroix et al. 2004)
	Ammonium transporter AmtB	PL	Mass spectroscopy	(Patrick et al. 2018)
	SERCA	PL	ATPase activity—radiometric ligand binding assays	(Lee 2011)
	Na^{+}, K^{+}-ATPase	PL	ATPase/p-nitrophenyl phosphatase activity	(Ottolenghi 1979; Santos et al. 2005)
	ZntA ATPase	PL	ATPase activity	(Zimmer and Doyle 2006)
Receptor	P2X7	Proton	Patch clamp	(Virginio et al. 1997; Flittiger et al. 2010)
	Adenosine A2A receptor	Cho	GTPase activity	(Huang et al. 2022)
	Bacteriorhodopsin (bR)	PL	Folding yield follow by absorption spectroscopy	(Allen et al. 2004)
	OXTR	Cho	Radiometric and fluorescence ligand binding assays	(Gimpl et al. 1995; Muth et al. 2011)
Other	SecYEG complex	PL	ATPase activity	(Van Der Does et al. 2000; Koch et al. 2019)
	Cytochrome oxidase	PL	Oxidase activity	(Yu et al. 1975; Öjemyr et al. 2012)

PL, phospholipids; *Cho*, cholesterol

approach was fruitful to get knowledge on the regulation of membrane proteins by unusual ligands such as lipids or protons are P-type ATPases and PIP aquaporins.

Cooperative regulation of the enzyme activity of P-type ATPases by phospholipids

Early reports show that phospholipids are critical for the development of enzyme activity on different P-type ATPases (Levi et al. 2000; Filomatori and Rega 2003; Lee 2011). P-ATPases constitute a large group of integral membrane proteins involved in active transport across the cell membrane coupled to the hydrolysis of ATP (Dyla et al. 2020). This family shares common structural features including a large transmembrane domain (M) and three or more cytoplasmic domains (Fig. 1a): the so-called actuator (A) domain, the phosphorylation (P) domain, and the nucleotide binding (N) domain. Together, P and N constitute the catalytic domain, which contains all the machinery to bind and catalyze the hydrolysis of ATP. The transmembrane domain is composed by 6–10 α-helices arranged in a helix bundle (Fig. 1b) defining a "pathway" for the transported species (usually ions) through the hydrophobic environment of the membrane. These membrane proteins are mostly reconstituted in mixed micelles of phospholipids and detergent for structural and functional studies and require a minimum amount of lipids to be fully active (Hossain and Clarke 2019).

Mixed micelles constitute a good model for studying interactions between membrane proteins and phospholipids. Compared to bilayers, mixed micelles offer a simplified system where several physical forces, such as lateral pressure, are considerably weaker (Zhou & Cross 2013; Ratkeviciute et al. 2021). An illustrative example is the regulation of GIRK channels by PIP2. Wang et al. (2014) observed a cooperative response in bilayers, while similar experiments performed in micelles showed a hyperbolic curve (Niu et al. 2020). Earlier research by Rober Cantor demonstrated that changes in lateral pressure can elicit a sigmoidal response in membrane proteins (Cantor 1999a, 1999b). Therefore, mixed micelles represent a more suitable experimental model for analyzing the *chemical part* of the regulation process. Furthermore, in P-ATPases, it has been demonstrated that the catalytic activity and its regulation by natural ligands are retained when the protein is extracted from the cellular membrane and reconstituted in mixed micelles (Kosk-Kosicka 1990).

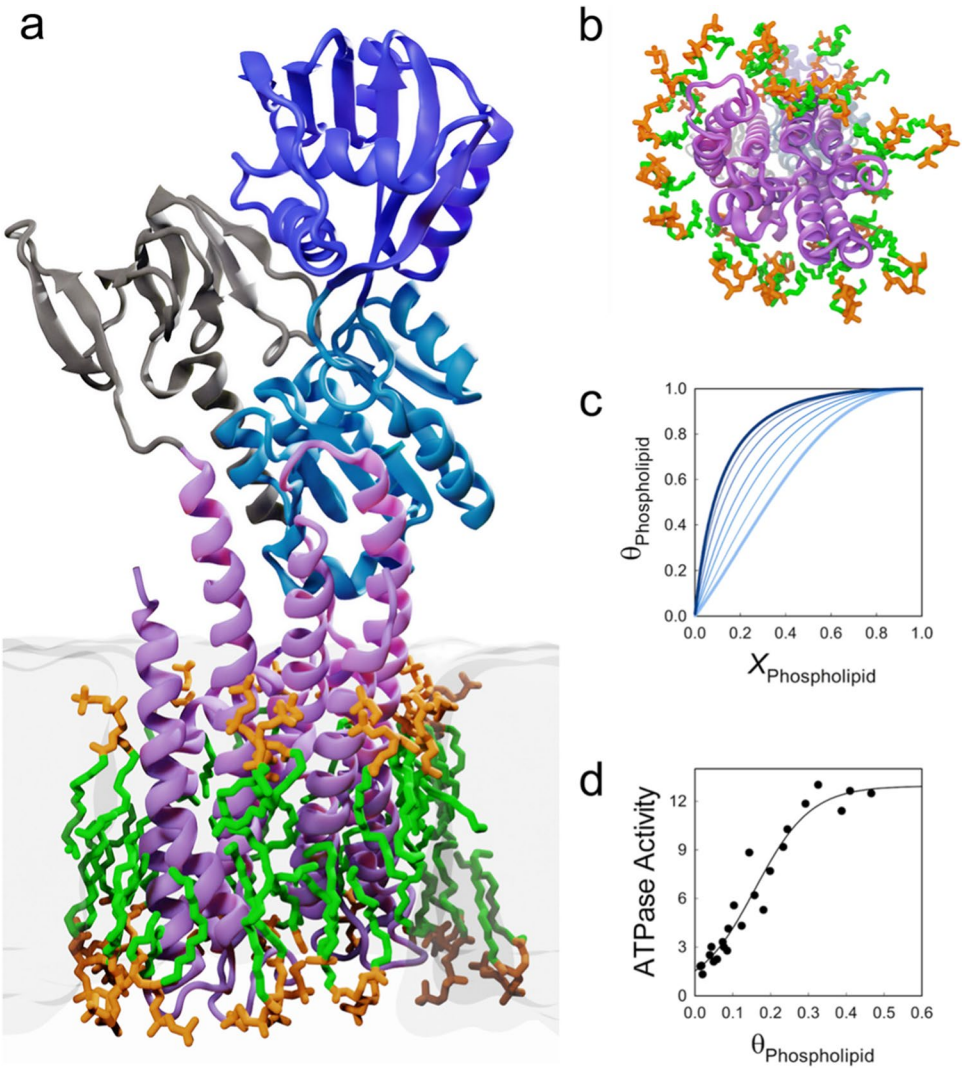

Fig. 1 General structure of P-type ATPases, viewed from a vertical cross-sectional plane of the membrane (**a**) and the luminal side (**b**). The cytoplasmic catalytic domain is color-coded in cyan (P subdomain) and blue (N subdomain) and the actuator domain is depicted in gray, while the transmembrane region is shown in bright lavender. Additionally, the surrounding lipids are depicted in green (hydrophobic tail) and orange (polar heads). Panel (**c**) displays simulated curves for the fractional coverage of the transmembrane surface (θ_{PL}) and the molar fraction of phospholipids (X_{PL}), using different phospholipid-detergent exchange constants (K_{ex}): 1.4, 2, 3, 5, and 10 (blue lines with increasing color saturation). Thicker lines, corresponding to the lowest or highest K_{ex} values, are the simulated curves using the experimental exchange constant between phosphatidylcholine and the detergent $C_{12}E_{10}$ ($K_{ex} = 1.4$) and between soybean phospholipids and $C_{12}E_{10}$ ($K_{ex} = 10$) on the transmembrane surface of the plasma membrane calcium pump (PMCA) (Levi et al. 2003). Panel (**d**) shows the PMCA maximal ATPase activity dependence on θ_{PL} (adapted from Dodes Traian et al. 2012)

When the plasma membrane calcium pump (PMCA) was reconstituted in mixed micelles containing increasing amounts of phospholipids, a cooperative enhancement in the enzyme's maximal ATPase activity was detected. The experimental design incorporated the well-established stabilizing effect of phospholipids on membrane proteins (Bowie 2001; González Flecha 2017), an effect that has been well characterized in the case of PMCA (Levi et al. 2000, 2002). This knowledge was critical in designing the experiments to prevent any significant thermal inactivation of the enzyme under all tested conditions. The phospholipid-induced activation of PMCA was reversible and depended on the phospholipid/detergent mole ratio and not on the total lipid concentration. Interestingly, it was shown by 1-aniline-8-naphtalenesulfonate fluorescence that enzyme activation was accompanied by a closer packing of the transmembrane domain (Dodes Traian et al. 2012). Further increase in the phospholipid/detergent mole ratio results in a decrease in the ATPase activity probably related to the coexistence of micellar structures of high order, bicelles and vesicles (Pignataro et al. 2015).

In order to quantitatively describe the activation effect of phospholipids on the enzyme activity of this protein, a phenomenological model considering two time-separated stages was proposed. The first stage assumes the exchange equilibrium among amphiphiles (phospholipid and detergent molecules) at the hydrophobic transmembrane surface of the protein and the bulk micellar phase. The second stage consists in an amphiphile-induced conformational change that should be initially restricted to the transmembrane region of the protein, but it has to be further propagated towards the catalytic domain to produce either activation or inhibition of the enzyme. The experimental evidence about the presence of motionally restricted amphiphiles surrounding the transmembrane region of membrane proteins supports the first stage (Marty et al. 2016). For the analysis, we define θ_i as the fraction of the protein transmembrane surface covered by a given amphiphile:

$$\theta_i = \frac{\text{number of contact sites occupied by the amphiphile i}}{\text{total number of contact sites on the transmembrane surface}} \quad (4)$$

A simple Langmuir-type adsorption model allowed to explain the experimental data (Levi et al. 2003). In this model, the monolayer of motionally restricted amphiphiles surrounding the transmembrane domain of the protein, which constitute the effective nano-environment sensed by the protein, can be known for a given micellar phase composition if we know the adsorption exchange constant between each phospholipid and a reference amphiphile (the detergent in the case of mixed micelles, or phosphatidylcholine in the case of bilayers). This exchange constant can be measured by several methods being the most used electronic paramagnetic resonance (EPR) (Marsh 2008) and Förster resonance energy transfer (FRET) (Levi et al. 2003; Loura et al. 2010). Figure 1c shows that a nonlinear relationship between the boundary monolayer composition and the bulk composition of the micellar phase is evident even for phospholipids with low exchange constant values. This could be the reason behind the experimentally observed cooperative effect. However**,** it can be noted that the sigmoidal character of phospholipid activation is still present when the activity is represented as a function of the fractional coverage of the hydrophobic transmembrane surface by phospholipids (Fig. 1d). This cooperative-like behavior can be phenomenologically described by either a Hill equation or a differential logistic function. This last phenomenological approach was used to analyze the activation effects of phospholipids on the catalytic activity of several P-type ATPases: the human plasma membrane calcium pump PMCA (Dodes Traian et al. 2012; Pignataro et al. 2015), and two Cu(I) transport ATPases *Af*CopA from *Archaeoglobus fulgidus* (Bredeston and González Flecha 2016) and *Lp*CopA from *Legionella pneumophila* (Placenti et al. 2022). The maximum activation (A_{max}) values were characteristic of each protein and, for a given headgroup (e.g., choline), it depended on the length of the phospholipid acyl chain being higher for PMCA with DMPC (Pignataro et al. 2015). This result points to an optimal requirement for hydrophobic coverage. On the other hand, using the same phospholipid/detergent mixture (asolectin/$C_{12}E_{10}$), it was shown that all the analyzed ATPases have similar half-maximal parameter values ($X_{0.5}$ around 0.1 corresponding, for this mixture of amphiphiles, to a $\theta_{0.5}$ of 0.3) indicating that all they need a similar degree of coverage by phospholipids the reach the half-maximal activity. Finally, there were differences in the observed values of parameter *c* which represents an empirical cooperativity coefficient related to the steepest relative change in the enzyme activity. *Lp*CopA has the higher value of *c* (Placenti et al. 2022), thus presenting higher sensitivity to the increase in phospholipid coverage of the transmembrane surface, i.e., a more cooperative conformational transduction step. Conversely, the lower value of *c* corresponded to *Af*CopA (Bredeston and González Flecha 2016; Recoulat Angelini 2020), which seems logical because the natural membrane phospholipids of archaea (with highly methylated isoprenoid chains ether-linked to a glycerol-1-phosphate backbone forming diether or tetraether structures) are chemically very different to that present in other domains of life.

Cooperative regulation of PIP aquaporins water transport by pH

Aquaporins (AQP) are members of the Major Intrinsic Proteins (MIPs) family of membrane channels. The canonical function of this family is to be water channels, but today, the diversity of solutes reported to permeate different AQP isoforms suggest that it is a multifunctional family. Structurally, AQP are tetramers in which each protomer presents a functional pore (Fig. 2a and b). Thus, the quaternary conformation of each tetrameric AQP contains four pores. Although most AQP are constitutively open channels, some members of this family are gated by protons. Some groups of AQP are modulated by external pH, while others are known to be affected by cytosolic pH variations. Among the externally regulated AQP are mammal AQP0 (Németh-Cahalan et al. 2000, 2004; Virkki et al. 2001; Chauvigné et al. 2015), AQP3 (Zeuthen and Klaerke 1999; de Almeida et al. 2016; MacIver et al. 2009; Zelenina et al. 2003), AQP5 (Rodrigues et al. 2016), AQP6 (Ikeda et al. 2002; Yasui et al. 1999), AQP7 (Rothert et al. 2017; Katano et al. 2014; Mósca et al. 2018), and AQP9 (Rothert et al. 2017; Gotfryd et al. 2018) and among internally regulated AQP are human AQP4 (Kaptan et al. 2015) and plant PIP (Gebeau et al. 2002; Tournaire-Roux et al. 2003; Alleva et al. 2006). In some cases, sensor residues have been identified but without excluding the participation of other titrable residues. This is the case of the plant PIP channels where a histidine residue was identified as the main sensor of changes in internal pH and additional charged residues, such as aspartic acid and arginine, near the histidine residue were found to also affect the gating process but in a weak way.

PIP channels are the major water pathway in plant plasma membranes and show a sigmoidal proton dose–response curve for water permeability (Fig. 2c) suggesting that water transport through these channels is a cooperatively regulated phenomenon (Alleva et al. 2006; Verdoucq et al. 2008; Bellati et al. 2010; Jozefkowicz et al. 2016). Structural studies revealed that PIP AQP present a rearrangement of the intracellular loopD (Fig. 1a and 1b) when transits from open to closed conformation that promotes the moving of a key leucine residue inside the conducting pore blocking water transport (Frick et al. 2013; Canessa Fortuna et al. 2019).

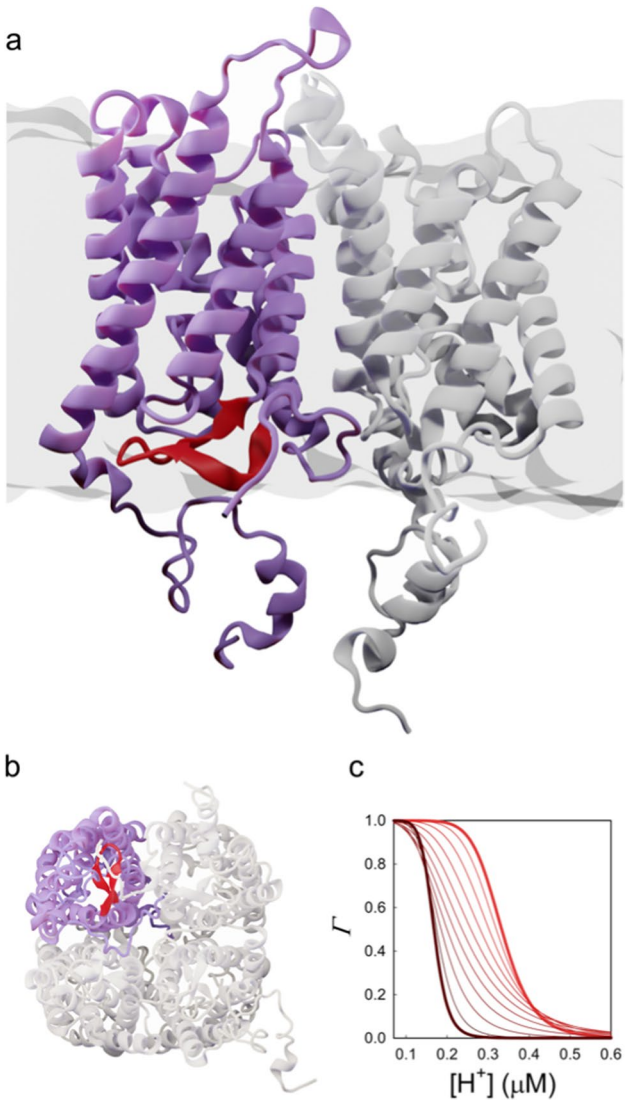

Fig. 2 General structure of aquaporins as viewed from a vertical cross-sectional plane of the membrane (**a**) and the cytosolic side (**b**). Aquaporins are organized as tetramers. A single protomer is depicted in bright lavender and the pH sensing region (loopD) is shown in red. **c** Simulated curves of the normalized water permeability (Γ) and the molar concentration of protons under different molar fraction of PIP1 or PIP2 protomers, ranging from 0 to 1 (adapted from Vitali et al. 2019). Thicker lines correspond to the maximum (dark red line) or minimum (red line) of PIP1 protomers in heterotetramers in the membrane

The PIP subfamily include two paralogues, PIP1 and PIP2, with high sequence similarity but some important functional differences, mainly regarding its ability to form homotetramers: while PIP2 channels are classical homotetrameric water channel, most PIP1 do not form homotetramers (Zelazny et al. 2007; Jozefkowicz et al. 2017) but can be part of heterotetrameric assemblies with PIP2 in a variable stoichiometry (Yaneff et al. 2014; Berny et al. 2016; Jozefkowicz et al. 2016). LoopD found in PIP2 and PIP1 are highly similar in sequence and both contain the histidine sensor residue, so all PIP2-PIP1 heterotetramers conserve pH sensitivity. However, pH regulation in homo vs heterotetramers, while sigmoidal, shows different $pH_{0.5}$. In the case of co-expression of PIP2 homotetramers and PIP2-PIP1 heterotetramers, an apparent cooperative response due to the different biological activity under the variation of intracellular proton concentration of both kinds of oligomers was found (Jozefkowicz et al. 2016; illustrated in Fig. 1c).

Although there is neither complete information on the parameters that govern the process of proton binding to the key loopD histidine residues in PIP1 or PIP2 protomers, nor is it certain that those residues are the only ones involved in the gating process, all the structural studies suggest that there are two stages involved in the process: PIP protonation and conformational reorganization. Phenomenological modelization of time-separated stages was used to analyze the impact of acidification in these channels, considering both the proton binding event and the open-closed conformational transition as putatively responsible for the cooperative behavior (Vitali et al. 2019). In this way, two different two-stage models were proposed accounting for PIP biological activity regulation to gain insight into the cooperativity of *Beta vulgaris* PIP2 homotetramers and *Beta vulgaris* PIP2-PIP1 (2:2) heterotetramers. One model considered that the cooperative step was the binding of protons (by using the Hill equation), being the subsequent conformational transitions independent for each protomer, and the other assumed that the proton binding is a non-cooperative process and that the subsequent conformational rearrangement, or transduction event, was cooperative (by using the logistic function). Both models accurately described the biological activity of the two molecular species assayed, PIP2 homotetramers and PIP2-PIP1 heterotetramers showing that cooperativity in these channels is not necessarily a consequence of cooperative proton binding as was usually considered, but it could also be the outcome of a cooperative conformational transition between open and closed states of the channel.

The study of the biological impact of co-expression of different stoichiometric ensembles of PIP isoforms in a same cell membrane is challenging since the expression of all these molecular species can have a clear impact in cell water transport. PIP isoforms have been shown to form hetero-oligomers with variable stoichiometry that depends on the expression level of each isoform and different heterotetramers can be expressed at the same time in a membrane (Jozefkowicz et al. 2016; Yaneff et al. 2014). Phenomenological modelization of pH regulated water transport for the co-expression of PIP2 homotetramers and PIP2-PIP1 heterotetramers allowed us to disclose the apparent cooperative response and estimate parameters for the individual molecular species involved in the biological response (i.e., PIP homotetramers, PIP2-PIP1 2:2 heterotetramers and

PIP2:PIP1 3:1 heterotetramers) (Vitali et al. 2019). The mathematical description of the process yielded confident values for phenomenological parameters describing the degree of cooperativity for both PIP2 homotetramers and PIP2-PIP1 heterotetramers, and showed that their cooperativity was not significantly different.

While each type of PIP oligomer (PIP2 homotetramer or any PIP2-PIP1 heterotetramer) has a strong cooperative response to pH, the different $pH_{0.5}$ of each ensemble give rise to a spectrum of apparent low cooperativity depending on the relative expression of each molecular species (Fig. 2c). It was demonstrated that the experimentally determined cooperativity for PIP channels can be masking real cooperative degrees of each kind of oligomeric form when isoforms with quite different proton sensitivity are co-expressed. This level of regulation combined with the lack of mechanistic data regarding proton binding in an oligomeric membrane protein is clearly benefited by phenomenological approaches.

Final considerations

Cooperativity is a long-studied kind of regulatory process that modulates biological activity of proteins. From a biophysical point of view, the elucidation of cooperative mechanisms is a goal of many investigations. Unfortunately, in some cases of biological relevance, structural or dynamic information regarding ligand binding sites is incomplete or even not available. Some of these cases involve integral membrane proteins regulation by protons and lipids (Table 1). Here, we make a detailed review of two cases where cooperative regulation was not possible to be studied by a mechanistic approach: phospholipid regulation of PMCA and proton regulation of homo and heterotetrameric PIP aquaporins ensembles. In both cases, phenomenological models were useful to describe experimental data and to expand our understanding about function regulation of both types of membrane proteins.

Phenomenological models have been, and continue to be, significantly valuable for the advance of knowledge across diverse research fields. Recent studies have showcased their effectiveness in various areas, including the exploration of amphiphilic peptide translocation through membranes (Bartoš et al. 2021), the examination of the pH-dependent aggregation of intrinsically disordered proteins (Santos et al. 2020; Pintado et al. 2021), the determination of the directionality of allosteric responses within a protein (Loutchko and Flechsig 2020), the analysis of second-order topological insulators (Bernard et al. 2023), the study of electrodynamic features in dust storms (Zhang and Zhou 2023), and the investigation of force-dependent detachment rates in molecular motors (Dutta and Hana 2021). These instances showcase the significance of employing phenomenological models to enhance our comprehension of intricate phenomena. The last decades in biosciences have been denoted by the central role given to the mechanistic explanations of molecular phenomena. The preponderance of mechanistic proposals has been so overwhelming that it has impacted even in the philosophy of biological sciences where the *new mechanism* research program emerged (Machamer et al. 2000). Without denying the fruitfulness of the description of mechanisms and mechanistic explanations, we will underscore that phenomenological modeling still has a central epistemic value (as shown with the historical re-elaboration of the Hill equation and the two recent cases of study described, PIP channels and PMCA), mainly in all those cases where there are so many (unknown) variables that it is impossible to even sketch a mechanistic proposal.

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Declarations

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