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Impact of Quaternary Structure Dynamics on Allosteric Drug Discovery

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Abstract

The morphoein model of allosteric regulation draws attention to proteins that can exist as an equilibrium of functionally distinct assemblies where: one subunit conformation assembles into one multimer; a different subunit conformation assembles into a different multimer; and the various multimers are in a dynamic equilibrium whose position can be modulated by ligands that bind to a multimer-specific ligand binding site. The case study of porphobilinogen synthase (PBGS) illustrates how such an equilibrium holds lessons for disease mechanisms, drug discovery, understanding drug side effects, and identifying proteins wherein drug discovery efforts might focus on quaternary structure dynamics. The morphoein model of allostery has been proposed as applicable for a wide assortment of disease-associated proteins (Selwood, T., Jaffe, E., (2012) *Arch. Bioch. Biophys.* **519**:131–143). Herein we discuss quaternary structure dynamics aspects to drug discovery for the disease-associated putative morphoeins phenylalanine hydroxylase, HIV integrase, pyruvate kinase, and tumor necrosis factor α . Also highlighted is the quaternary structure equilibrium of transthyretin and successful drug discovery efforts focused on controlling its quaternary structure dynamics.

Keywords

HIV integrase; morphoein; phenylalanine hydroxylase; porphobilinogen synthase; pyruvate kinase; transthyretin; tumor necrosis factor alpha; protein dynamics

Quaternary structure dynamics is an emerging target for allosteric drug discovery with tremendous potential for future growth, though few established drug targets are currently being approached from this vantage point. Nevertheless, there is growing evidence that some drugs function as inhibitors by trapping an assembly that is one component of an equilibrium of assemblies whose interchange controls a fluctuation between essential normal protein functions. The focus of this article is predominantly on homo-multimeric proteins whose functions include or are controlled by assembly/disassembly equilibria. In some cases, an equilibrium of functionally distinct multimers self-assemble from alternate conformations of a protein subunit. The latter is characteristic of proteins that use the morphoein model of allosteric regulation [1–3], illustrated schematically in Figure 1. We recently identified ~40 proteins whose behavior suggests such an allosteric mechanism [1]. Eight are directly linked to causation or exacerbation of various inborn errors of metabolism (e.g. phenylketonuria, ALAD porphyria, E3-deficient maple syrup urine disease, CBS deficiency, hemolytic anemia, and ADSL related autism). Twenty-four are proteins whose function has a profound effect on metabolism and/or other cellular processes related to the initiation, promotion, and progression of cancer (e.g. pyruvate kinase, peroxiredoxin, p53; dihydrolipoamide dehydrogenase, geranyl geranylpyrophosphate synthase; RNase A). Others are chemotherapeutic targets for infectious diseases (porphobilinogen synthase, HIV

integrases). These are some examples where small molecule regulation of a quaternary structure equilibrium is a valid approach to drug discovery.

Background

Our interest in harnessing quaternary structure dynamics as a mechanism to control protein function arose from the unexpected behaviors of the essential enzyme uroporphobilinogen synthase (PBGS, a.k.a. 5-aminolevulinic acid dehydratase, E.C. 4.2.1.24), which catalyzes the first common step in the biosynthesis of all the tetrapyrrole cofactors such as heme, chlorophyll, vitamin B12, cofactor F430, as well as the linear bilins. PBGS participates in a quaternary structure equilibrium that can be summarized as $8mer \rightleftharpoons 2mer \rightleftharpoons 2mer^* \rightleftharpoons 6mer^*$, as illustrated in Figure 2 and recently reviewed [4–5]. The interconversion between 2mer and 2mer* is a hinge motion between two domains of each subunit; this motion is sterically forbidden within the context of 8mer or 6mer*. The octamer (8mer) is a high activity state, the dimer conformations 2mer and 2mer* are at a very low mole fraction (<0.5%), and the hexamer (6mer*) is a low activity state [6]. In wild type human PBGS at neutral pH, the mole fraction of 8mer is >95%; as the pH rises, the mole fraction of 6mer* increases to >95% by pH 9. This quaternary structure dynamic accounts for the pH dependent decrease in enzyme activity. The low activity and limited amount of 6mer* (<5%) present at neutral pH in wild type human PBGS obscured the existence of this multimer for many years. However, the past decade's work has revealed that 6mer* is a physiologically relevant assembly and that the PBGS quaternary structure dynamic behavior has specific implications for: 1) small molecule allosteric modulation of protein function (e.g. allosteric drug discovery); 2) the development of new antimicrobials; 3) understanding and treatment of an inborn error of metabolism; and 4) exposing the molecular basis for drug action including some off-target drug side effects.

Multimer-specific small molecule binding sites

A key aspect of small molecule modulation of the PBGS quaternary structure equilibria is the presence of multimer-specific surface cavities that can serve as small molecule binding sites. The PBGS multimers 8mer and 6mer* contain oligomer-specific surface cavities (Fig. 2b) that can act as binding sites for allosteric activators or inhibitors, respectively. Binding of an allosteric regulator to such a surface cavity stabilizes the regulator-bound assembly and promotes the function of said assembly. A variety of *in silico* and *in vitro* approaches allowed identification of small molecule allosteric regulators that could modulate PBGS function by stabilizing a specific multimer [7–10]. Species selective 6mer* stabilization, and thereby species selective enzyme inhibition, has been achieved – presumably because the oligomer-specific allosteric binding sites (surface cavities) are phylogenetically variable [9]. Thus, harnessing an intrinsic quaternary structure equilibrium for allosteric inhibition of a universally essential enzyme can be applicable to the search for new antimicrobial targets. Herein we consider whether harnessing similar quaternary structure equilibria of other homo-multimeric proteins is generally applicable to drug discovery efforts. The first such species-specific inhibitor of PBGS, which was specific for stabilizing the low activity 6mer* of a plant PBGS, was given the name morphlock-1 [9]. We propose the general term morphlock to describe a compound that modulates protein activity by specifically binding to and stabilizing one component of an equilibrium of morphoein forms (see Fig. 1a).

Oligomer-specific surface cavities form a rich canvas for the identification of small molecule allosteric effectors such as morphlocks. However, PBGS also provides an example of an oligomer-specific subunit-subunit interface that contributes directly to the distinct behaviors of the 8mer and 6mer* assemblies. Comparison of the PBGS multimers (Fig. 2a) reveals that 8mer contains an interface between an N-terminal arm domain of one subunit

and the perimeter of the $\alpha\beta$ -barrel domain of a neighboring subunit. This “hugging” interaction is not present in 2mer, 2mer*, or 6mer* and accounts for the ability of 8mer to stabilize a closed conformation of the enzyme active site lid to facilitate catalysis at neutral pH [4]. In the closed conformation, the active site lid interacts with one of the PBGS substrate molecules, thus substrate bound at the active site also stabilizes the closed lid conformation and consequently stabilizes 8mer [11]. The 8mer-specific subunit interface provides the binding site for an allosteric magnesium ion, which is present in almost all PBGS except those derived from metazoa (animals), and fungi [12]. Figure 3 illustrated this phylogenetically variable allosteric metal ion binding site and the effect of magnesium on the equilibrium between 6mer* and 8mer of a plant PBGS. As originally noted by Monod and coworkers, allostery provides control mechanism that are independent of active site conservation, and PBGS is a prime example of this. In the case of PBGS: 1) the multimer-specific surface cavities are of phylogenetically variable amino acid composition; 2) the mechanisms for controlling the quaternary structure equilibrium are phylogenetically variable (see Fig. 3); and 3) some pathogens have evolved protein structure components that forbid sampling the 2mer*/6mer* half of the quaternary structure equilibrium (Fig. 2c).

Morpheesins and inborn errors of metabolism

A severe lack of PBGS activity leads to the rare inborn error of metabolism known as ALAD porphyria. There are eight disease-associated PBGS variants located throughout the protein structure, all of which shift the quaternary structure equilibrium toward 6mer* [13]. Thus, perturbation of an intrinsic dissociative quaternary structure equilibrium is one type of conformational disease, in this case an inborn error of metabolism. Small molecule allosteric activators that function by stabilizing the PBGS 8mer could provide a therapeutic for ALAD porphyria, though no such allosteric activator has yet been identified. We are currently using a docking approach to find 8mer-stabilizing molecules that bind to the 8mer-specific cavity illustrated in Figure 2b. In addition to PBGS, the putative morpheesins whose dysfunction is related to an inborn error of metabolism are adenylosuccinate lyase (EC 4.3.2.2), ATPase of the ABCA1 transporter, cystathionine β -synthase (EC 4.2.1.22), dihydrolipoamide dehydrogenase (EC 1.8.1.4), mitochondrial NAD(P)⁺ malic enzyme (EC 1.1.1.40), phenylalanine hydroxylase (PAH, EC 1.14.16.1), and pyruvate kinase (EC 2.7.1.40), the latter two of which we discuss below. In addition to PBGS, several other of the putative morpheesins related to inborn errors of metabolism are associated with one or more changes to single amino acids outside the enzyme active site. We have put forth the hypothesis that the active 8mer of PBGS is a “fragile” assembly [4] and that many single amino acid changes can perturb the quaternary structure equilibrium toward 6mer*. Single amino acid variants that dramatically alter the quaternary structure equilibrium of a protein suggest a fragile protein multimers consistent with the morpheein model of allosteric regulation.

Drugs and environmental contaminants act on the PBGS quaternary structure equilibrium

Pertinent to the unexpected effects of drugs and environmental contaminants, for which molecular mechanisms are often unknown, is the *in vitro* identification of allosteric modulators of PBGS from libraries of such compounds. Work with human PBGS suggests that some drugs work off target to inhibit PBGS by stabilizing the low activity hexameric assembly (6mer*) [7–8]. An *in vitro* screen of the ~1500 compound Johns Hopkins Clinical Compound Library revealed a dozen drugs that act as 6mer*-stabilizing inhibitors of human PBGS [7]. One such drug, diclofenac, is a common non-steroidal anti-inflammatory whose use is contraindicated in porphyric patients by the American Porphyria Foundation. We have posited that small molecule 6mer* stabilization of human PBGS may contribute to the episodic nature of ALAD-porphyria and other porphyric illnesses. Subclinical effects in the

normal population could easily be amplified in carriers of the ALAD porphyria-associated alleles or in those suffering from other clinical conditions that result in PBGS inhibition, such as lead poisoning [14]. ALAD porphyria associated variants were shown to be more sensitive to 6mer* stabilization by these drugs [7]. An *in vitro* screen of the National Toxicology Program's ~1400 compound library of environmental contaminants also revealed a dozen compounds that stabilize the human PBGS 6mer*, the most potent of which is a ubiquitous contaminant in chlorinated drinking water known as mutagen X [10].

The identification of drug targets whose allosteric mechanism may involve dissociative quaternary structure equilibria

Our work with PBGS sets a precedent for: 1) looking to universal metabolic pathways for opportunities for new antimicrobial targets; 2) looking to quaternary structure equilibrium perturbation as a molecular mechanism for inborn errors of metabolism and the treatment thereof; and 3) considering the perturbation of an intrinsic dissociative quaternary structure equilibrium as a mechanism for drug action, drug side effects, and toxin action. The first step in the application of these ideas to drug discovery is the identification of proteins (putative drug targets) whose function is governed by an equilibrium of alternative quaternary structure assemblies.

To date PBGS is the only protein whose allostery is definitively based in an equilibrium between architecturally and functionally distinct multimers whose interconversion *must* include multimer dissociation and a conformational change in the dissociated state. Hence, PBGS is the prototype morphoein, a term we coined to describe proteins that use the morphoein model of allostery, generally depicted in Figure 1. We have published extensively on protein characteristics that suggest the identification of a protein as a morphoein [1–2, 11, 15–16]. However, these “morphoein-suggestive” characteristics are *behavioral* (e.g. one band on an SDS PAGE but multiple bands by native PAGE; a homogeneous protein that separates into multiple components on ion exchange or size exclusion columns; kinetic hysteresis, rate vs. substrate dependence that fits to a double hyperbolic equation, order-of-addition kinetic anomalies). Many of these anomalous behaviors are not associated with an unambiguous simple and searchable keyword or phrase, and are often described in text, footnotes, or discussion rather than the freely accessible abstract of a publication. Thus, identification of putative morphoeins from the literature has proven to be non-trivial. To date most of the identified putative morphoeins derive from reading the primary literature with emphasis on alternate data interpretation in light of a possible equilibrium of architecturally and functionally distinct multimers. Taking this approach reveals a plethora of suggestive evidence in the published literature, but very few published experiments that directly address whether oligomer dissociation is a *necessary* component of the allosteric mechanism. By exception, one recent publication explicitly identifies *Escherichia coli* glucosamine-6P synthase as using the morphoein model for allostery [17].

Unfortunately, none of the morphoein-suggestive characteristics are based in specific protein sequence-based information. Hence, powerful sequence-based informatics approaches to the identification of putative morphoeins, though conceptually possible, remain to be developed. In addition to the published descriptions of the behavioral aspects of putative morphoeins, we propose that there are also structural commonalities. For PBGS the essential conformational change alters the orientation between two domains of each subunit. Hinge motion between multiple folded domains is also an aspect of our views of phenylalanine hydroxylase and HIV integrase described below. For PBGS, multimer dissociation occurs along a subunit-subunit interface that is largely hydrophilic and where the crystal structures show phylogenetically invariant locations for intersubunit water molecules [6]. If multimer

dissociation is physiologically significant in an aqueous environment, then dissociation along hydrophilic interfaces must be thermodynamically feasible.

Examples where quaternary structure dynamics is proven or proposed to be an important aspect of drug action

Our focus on the protein “drug targets” described below derives largely from the aforementioned literature evaluations and reinterpretations. Not all of the chosen examples are among our published list of putative morphoeins [1], but all involve homo-multimeric proteins whose function responds to an equilibrium between alternate quaternary structure assemblies and for which control of this equilibrium is proposed as a target for drug discovery. Below we discuss the quaternary structure dynamics of five proteins wherein such dynamics are associated with mechanisms for drug action or drug discovery. The first is phenylalanine hydroxylase where a newly proposed morphoein model suggests the existence of an oligomer-specific subunit-subunit interface, akin to the allosteric binding site for the magnesium of PBGS, which serves as the allosteric binding site for the physiologic activator phenylalanine [18]. Aspects of this model suggest a new approach to the development of pharmacologic chaperones for a subset of PKU patients. The second is HIV integrase where two independent groups, intending to disrupt essential interactions between integrase and another protein, instead found effective inhibitors that function by stabilizing one morphoein form of the integrase quaternary structure equilibrium [19–20]. The third example is an isozyme of pyruvate kinase, where the cancer promoting splice variant PKM2 has been shown to have alternate assemblies with alternate (moonlighting) functions and the modulation of this equilibrium holds promise for new cancer drugs [21–22]. The fourth is the oligomeric TNF α , where a potent inhibitor was found to stabilize an alternate multimeric form [23]. The last example, and the only one related to amyloid disease, is transthyretin, where modulation of the multimer equilibrium has been shown effective against amyloid formation [24]. In this last example, a quaternary structure equilibrium is clearly the target for drug discovery, but it is unclear if this fits the classic definition of allostery.

Stabilization of phenylalanine hydroxylase (PAH) as a therapeutic approach to phenylketonuria (PKU)

Human PAH dysfunction is one of the most common inborn errors of metabolism. Untreated prior to the mid-20th century, unregulated blood phenylalanine levels and the resultant PKU filled institutions with severely mentally and physically disabled patients. Protein-limited diets have largely cured the severe neurologic disabilities. However, alternative therapeutics are needed for lifelong control of blood phenylalanine levels to prevent patients from suffering defects in executive function and behavior [25–27]. One such therapeutic approach derives from the characterization of PKU-associated PAH variants as defective in protein folding or stability [28]. There are, in fact, many hundreds of PKU-associated PAH variants, most of which are not at the enzyme active site, but which still perturb the proper functioning of PAH [29]. Based on the extensive literature and structural information of PAH, we consider an alternate view that the improper functioning of some PKU-associated PAH variants derives from an altered quaternary structure equilibrium. This view focuses on the *assembly* of multiple subunits rather than the *folding* of individual subunits.

Human PAH is an allosteric protein characterized by a complex kinetic and quaternary structure behavior that is consistent with a morphoein model of allosteric regulation [30–31]. In this model, and consistent with published literature, phenylalanine is both a substrate binding at the enzyme active site, and an allosteric activator binding to the regulatory

domain in such a way as to stabilize a high activity tetramer. Allosteric activation by phenylalanine allows the body to amplify PAH activity and prevent high phenylalanine concentrations from blocking tyrosine and tryptophan import into the brain, where these amino acids are precursors to important neurotransmitters. The proposed PAH quaternary structure equilibrium can be summarized as $4mer^* \rightleftharpoons 2mer^* \rightleftharpoons 2mer \rightleftharpoons 4mer$, illustrated schematically in Fig. 4, where 4mer is a high activity tetramer and 4mer* is a tetramer with basal activity [18]. The limited activity of the nmer* components derives from a portion of the regulatory domain being able to sample a conformation that blocks the enzyme active site, as is seen in one crystal structure of rat PAH [32]. Learning from our extensive characterization of PBGS, the key transition between dimers ($2mer \rightleftharpoons 2mer^*$) is proposed to reflect a reorientation of two domains of each PAH subunit; the difference between the forms with basal activity and the high activity form reflect differences in active site access. Thus, the nmer components are proposed to rotate the regulatory domain relative to the catalytic domain and disallow sampling the inhibitory active site-blocking interaction. PAH has three domains (see Fig. 4). Like many multidomain proteins, there are X-ray crystal structures of one and two domain constructs, but not of the full-length three domain protein. We have postulated that difficulty in obtaining crystal structures of full length multidomain proteins may arise from the sample heterogeneity created by a physiologically significant equilibrium of alternate assemblies [3]. Key to the future development of allosteric pharmacologic chaperones for PAH is the discovery of small molecules that specifically bind to and stabilize 4mer, presumably to a 4mer-specific binding site that does not compete with the allosteric phenylalanine binding site. Interestingly, one PAH therapeutic currently in use is the PAH cofactor tetrahydrobiopterin, which binds at the enzyme active site (not allosterically) and is believed to work by structurally stabilizing PAH subunits. Currently marketed as Kuvan, this therapeutic is quite effective for a subset of patients. We propose that Kuvan may function by stabilizing 4mer* or 2mer* and thus bringing PAH of lower intrinsic stability into the equilibrium of quaternary structure isoforms which can then sample the higher activity multimers.

Multimer stabilizing inhibitors of HIV integrase

Retroviral integrases such as HIV integrase (IN) are responsible for two temporally distinct catalytic reactions, which are the 3' end processing of viral DNA (removing a specific dinucleotide from each of the viral DNA ends), and a concerted cleavage and ligation reaction that joins the new viral 3'ends to staggered cuts in the host genomic DNA. The two reactions, which take place in the same active site, together are referred to as integration. For human HIV IN, preferred sites of integration are mediated through complex formation with a cellular factor, LEDGF/p75. A state-of-the-art view of IN function includes an incompletely defined multimeric equilibrium of integrase assemblies including monomers, dimers, and tetramers where a dimer is sufficient for the processing reaction, but a tetramer is required for the concerted integration of two viral DNA ends. Like many proteins, each IN monomer comprises multiple domains, each of which folds into a well defined tertiary structure, with linker regions connecting them. The three domains of IN are in the order N-terminal domain, catalytic core domain, and C-terminal domain. Recent analysis of IN-containing crystal structures suggests that the monomer subunits assemble in more than one way, yielding dimers of differing architecture [33]; a combination of chemical cross linking and small angle X-ray scattering studies reveal that there is more than one way that IN subunits can assemble into dimers [34]. In one such dimer, the core domains interact with each other; in the other dimer, called a reaching dimer, this interaction is not present and the two terminal domains play a critical role. Either of these two dimer forms can assemble into a tetramer in the absence of DNA. However, the apo-IN tetramer architecture (Bojja et al., personal communication) is different than that of the tetramer bound to viral DNA. In the latter, one dimer form (the inner dimer), which is similar to the reaching dimer, binds and

acts upon the DNA substrates; the second (outer dimer), in which the core domains interact, has no catalytic role [35]. Thus, IN appears to fit the definition of a morphoein wherein different conformations of a monomer can assemble into structurally and functionally distinct oligomers. In the case of IN, nucleic acid is an essential component of one or more of the alternate multimers. To date there are two examples of multimer-stabilizing inhibitors of HIV IN. In 2007 Friedler and coworkers introduced the concept that HIV-1 IN could be inhibited by shifting its oligomeric equilibrium [19]. Not unlike the aforementioned morpholocks, they introduced “shiftides” as a class of peptides that inhibit proteins by binding to and stabilizing a low activity multimer in an equilibrium of alternate protein assemblies. A shiftide consisting of a 10-amino acid sequence of LEDGF/p75 was shown to stabilize an inactive DNA-free IN tetramer and inhibit both the processing and integration reaction. Independent efforts to design small molecule IN inhibitors that bind in the LEDGF/p75 binding pocket resulted in the discovery of 2-(quinolin-3-yl) acetic acid derivatives, denoted LEDGINS, whose detailed mechanism of action was also unexpectedly found to be the trapping of an inactive IN multimer [20]. LEDGINS trap an IN dimer containing the core:core interface and disallow formation of an essential multimer. In so doing, LEDGINS inhibit viral DNA binding, the IN processing reaction, the integration reaction, and the production of infective virus particles. Thus, the remarkably potent LEDGINS are the product of a structure-based drug design effort directed at interfering with the binding of LEDGF/p75 by IN, but result in an inhibitor whose potency is driven by its ability to modulating the exchange of IN between alternate multimers.

The publication introducing shiftides discussed that it is easier to design an inhibitor to bind to and consequently stabilize an inactive multimer than it is to design an inhibitor that interferes with multimerization [19]. In fact, this has been our experience with efforts to perturb the PBGS quaternary structure equilibrium. In our hands, each approach to indentifying compounds that stabilize a higher order multimer have been successful at finding multimer-stabilizing compounds that act allosterically to alter (in this case, inhibit) enzyme activity [7–10]. However, antimicrobial efforts directed at PBGS that do not sample the hexameric assembly (e.g. see Fig. 1c) were instead focused on finding small molecules that would prevent assembly of the pro-octamer dimer to the octamer. These efforts, which did find compounds that would inhibit 8mer formation, did not result in compounds that could compete with the 8mer-stabilizing effect of substrate [36–37]. Thus, these dimer-stabilizing compounds did not prevent substrate induced formation of 8mer and did not act as inhibitors.

Multimer stabilization of pyruvate kinase

Pyruvate kinase stands at a central position in energy metabolism, transferring a phosphoryl group between phosphoenol pyruvate (PEP) and ADP. As recently reviewed, a splice variant of the muscle isozyme, PKM2, which is not associated with an inborn error of metabolism, has long been associated with cancer [38]. Although all pyruvate kinase isozymes exist primarily as homotetramers, PKM2 has an enhanced propensity to sample a dimeric assembly, which does not convert PEP to pyruvate in the presence of ADP; this dimeric assembly is less prevalent in PKM1 and the liver /red blood cell isozymes,. The rationale for an inactive pyruvate kinase supporting tumor growth is to direct glycolytic intermediates (e.g. pyruvate) to biosynthetic pathways. However valid this rationale, it also appears that dimeric PKM2 gets translocated to the nucleus and acts in gene transcription regulation. An important recent paper strongly supports the hypothesis that tetrameric PKM2 can function in glycolysis while the dimeric form of PKM2 can transfer a phosphoryl group between PEP and a specific tyrosine residue of the transcription factor stat3 [22]. This has been referred to as a protein kinase moonlighting activity of PKM2. However, as a nucleotide (e.g. ATP) is not the phosphoryl donor, this may be another example where commonly understood

enzyme nomenclature lacks precision. Regardless of the name, the PKM2 catalyzed PEP-phosphotransferase reaction occurs in the cell nucleus where phospho-stat3 can perform its transcription activation. Thus, one would predict that small molecule stabilization of tetrameric PKM2 could form the basis for a drug that would have antiproliferative effects. Dynamix Pharmaceuticals reports a program to develop small molecules that will stabilize the PKM2 tetramer and function to minimize the well known Warburg effect seen in tumors (<http://www.dynxp.com/pipeline/cancer-metabolism/pkm2>). PKM2 tetramer stabilization is expected to have this physiological effect as well as limiting the ability of PKM2 to travel into the nucleus and act as a protein kinase.

Multimer stabilizing inhibitors of TNF α

Tumor necrosis factor alpha (TNF α) has a role in inflammatory diseases and is a well established drug target. TNF α is a trimeric protein and only a very small portion of the literature considers alternate multimers (e.g. [39–40]). However, drug discovery efforts at Sunesis Pharmaceuticals resulted in a small molecule inhibitor whose TNF α -bound X-ray crystal structure unexpectedly showed the inhibitor bound to a TNF α *dimer* and interacting with subunit-subunit interface residues that are not surface accessible in the crystal structure of the TNF α trimer [23]. The inhibitor is shown to bind to a conformation of the TNF α dimer that has a broadened angle of subunit-subunit interaction and is thus not competent to form a trimer. Experimental data are presented as supporting a predissociation-independent model wherein the inhibitor binds to the TNF α trimer whose breathing motions allow inhibitor to access the normally buried inhibitor binding site and thus promote expulsion of one subunit. However, in support of an alternative model where inhibitor binds to and stabilizes a transiently formed TNF α dimer, docking studies targeting the crystal structure of the TNF α dimer successfully identified a handful of TNF α inhibitors [41].

Multimer stabilization of transthyretin

Transthyretin functions as a tetramer to bind and transport the hormone thyroxine, which binds at a subunit-subunit interface. Native transthyretin tetramer dissociation is an essential component of the most common familial amyloidogenesis diseases. Kelly and coworkers discovered that certain non-steroidal anti-inflammatory drugs (NSAIDs) can inhibit amyloidogenesis by stabilizing the native tetramer [42]; these NSAIDs are structural analogs of thyroxine and bind at the subunit:subunit interface. Follow-up studies established that heterotetramers of transthyretin in familial amyloid polyneuropathy patients are significantly less stable (more likely to dissociate to monomers) than transthyretin of normal subjects [43]. The most effective transthyretin stabilizing NSAID is diflunisal; ongoing clinical trials show few recognized side effects normally associated with NSAIDs [44]. Clearly diflunisal functions to inhibit amyloid formation by stabilizing the transthyretin tetramer. However, since diflunisal binds at the thyroxine binding site, a question arises as to whether this inhibition is properly designated as allosteric.

Summary

PBGS provides an example wherein a dissociative equilibrium of functionally and architecturally distinct multimers provides a structural basis for allostery, for an inborn error of metabolism, and for drug action. PBGS constitutes the prototype morphecin and its behaviors suggest other proteins where perturbation of a dissociative quaternary structure equilibrium is a valid basis for small molecule modulation of protein function. As examples we highlight the behavior of PAH, HIV IN, PKM2, TNF α , and transthyretin, where all but the latter is a putative morphecin and the subunit structure does not undergo “refolding” on the level of tertiary structure as part of the disease. Instead the conformational changes involve alternate orientations of subunits and/or of domains within subunits. In the case of

HIV IN and TNF α , we highlight drug discovery efforts that unexpectedly resulted in the elucidation of an inhibitor that stabilizes a specific multimer and in doing so prevents normal protein function. In the case of PAH we present a new morpheein model for allosteric regulation that suggests a new approach to developing a therapeutic for PKU. In the case of PKM2 we review its alternate moonlighting functions, pyruvate kinase and PEP-protein phosphotransferase, which provides two mechanisms by which multimer stabilization could act against cell proliferation (cancer). In the case of transthyretin, multimer stabilization prevents accumulation of a monomer that can progress to amyloid. Only this last example is related to an amyloid disease. The future promises further application of quaternary structure dynamics to drug discovery. Particularly intriguing are the metabolic enzymes, like pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase [45], which have alternate functions in alternate cellular compartments, or whose reversible polymerization is associated with cell cycle control, such as inosine monophosphate dehydrogenase and CTP synthase [46], the former of which is also associated with moonlighting functions [47–48].

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Figure 1. Schematic representations of the morpheein model of allosteric regulation showing the interchange of alternate oligomers mediated by a conformational change in the dissociated state
a) A 2D geometric illustration includes a representation of the effect of allosteric regulators (morphlocks) on the position of the equilibrium. Oligomer assembly associates the dashed lines with the solid lines such that the pie wedge assembles into a trimer and the rectangle assembles into a tetramer. Allosteric effector (solid black shapes) binding stabilizes one or the other oligomer, thus drawing the equilibrium in the direction of the ligand-bound oligomer. **b)** A 3D morphing dice model where oligomer assembly associates a die face with one dot with a die face with four dots such that a six-sided cubic die assembles to a tetramer while a four-sided pyramidal die assembles to a pentamer. The cubes-to-pyramids conformational change involves the self-association of 5-dot surfaces with 6-dot surfaces within each individual subunit, perhaps via a hinge rotation between individual domains of each subunit.

Figure 2. The quaternary structure equilibrium of porphobilinogen synthase

a) The PBGS quaternary structure equilibrium includes an inactive hexamer (PDB id 1PV8) that does not have subunit interactions necessary for an ordered active site lid. Dissociation to the pro-hexamer dimer can be followed by a conformational change that reorients the two $\alpha\beta$ -barrel domains to form the pro-octamer dimer. Association of pro-octamer dimer to octamer (PDB id 1E51) includes formation of subunit interfaces that support order in the active site lid. **b)** Space filling representations, colored as in part **a**, illustrate several oligomer-specific surface cavities that can serve as allosteric regulator (drug) binding sites. A computational docking approach focused on the surface cavity indicated by the yellow circle successfully identified species selective allosteric inhibitors of both plant and human PBGS [8–9]. Hexamer stabilizing inhibitors of human PBGS were also discovered using *in vitro* screening methods; binding sites for these inhibitors have not yet been determined. **c)** PBGS from some human pathogens do not sample the hexameric assembly. *Toxoplasma gondii* PBGS (PDB id 3OBK, shown left) contains a 13 amino acid C-terminal extension that forms a domain swapped beta sheet (orange/yellow) that locks the dimer into the pro-octamer conformation. *Pseudomonas aeruginosa* PBGS (PDB id 1GZG, shown right) contains a monovalent cation (magenta) positioned to impede the conformational change between pro-octamer dimer and pro-hexamer dimer.

Figure 3. The phylogenetically variable allosteric magnesium of porphobilinogen synthase
 For all panels, chains are labeled as in the PDB file. **a)** The *E. coli* PBGS octamer (PDB id 1L6S) with the pro-octamer dimer in shades of green; the yellow circle highlights subunit-subunit interaction detailed in the enlargement. The octamer-stabilizing allosteric magnesium is shown as a magenta sphere with the five waters (cyan spheres) that comprise its first coordination sphere ligands, and the side chains of residues from chains A and B that also contribute to magnesium coordination. **b)** The human PBGS octamer (PDB id 1E51) with the pro-octamer dimer in shades of pink; the yellow circle highlights subunit-subunit interaction detailed in the enlargement. The side chain of Arg240 forms an 8mer-stabilizing hydrogen bond with the backbone carbonyl of Ser5 from its hugging dimer partner. **c)** The human PBGS hexamer (PDB id 1PV8) with the pro-hexamer dimer in shades of blue; the yellow circle highlights the displacement of the N-terminal arm. **d)** Native PAGE illustrating the transition of pea PBGS octamer to hexamer in the presence of EDTA [49].

Figure 4. A morphoein model for the allosteric regulation of PAH

A model for the allosteric regulation of PAH includes a dramatic reorganization of the regulatory domain relative to the catalytic domain [18]. In this model the 4mer* is of low activity because the regulatory domain partially blocks active site access. The 4mer, in addition to allowing active site access, contains a multimer-specific subunit-subunit interface. Molecules that can stabilize this interface are predicted to act as allosteric activators.