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Minireview

Cooperativity in monomeric enzymes with single ligand-binding sites

Carol M. Porter, Brian G. Miller *

Department of Chemistry and Biochemistry, 217 Dittmer Laboratory, Florida State University, Tallahassee, FL 32306-4390, United States

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ABSTRACT

Cooperativity is widespread in biology. It empowers a variety of regulatory mechanisms and impacts both the kinetic and thermodynamic properties of macromolecular systems. Traditionally, cooperativity is viewed as requiring the participation of multiple, spatially distinct binding sites that communicate via ligand-induced structural rearrangements; however, cooperativity requires neither multiple ligand binding events nor multimeric assemblies. An underappreciated manifestation of cooperativity has been observed in the non-Michaelis–Menten kinetic response of certain monomeric enzymes that possess only a single ligand-binding site. In this review, we present an overview of kinetic cooperativity in monomeric enzymes. We discuss the primary mechanisms postulated to give rise to monomeric cooperativity and highlight modern experimental methods that could offer new insights into the nature of this phenomenon. We conclude with an updated list of single subunit enzymes that are suspected of displaying cooperativity, and a discussion of the biological significance of this unique kinetic response.

Contents

1. Introduction

Cooperative interactions in biological systems are an inherent consequence of the dynamic nature of macromolecular assemblies. Structural rearrangements that accompany ligand binding provide a mechanism for long-distance communication between distant regions of a macromolecule. Cooperativity represents a manifestation

Abbreviations: LIST, ligand-induced slow transition; Itk, interleukin-2 tyrosine kinase; TIM, triosephosphate isomerase; Adk, adenylate kinase; CPMG, Carr–Purcell–Meiboom–Gill; FRET, Förster resonance energy transfer; smFPA, single-molecule fluorescence polarization anisotropy; TMR, tetramethylrhodamine; MODY, maturity-onset diabetes of the young; PHHI, persistent hyperinsulinemia of infancy.

* Corresponding author. Fax: +1 850 644 8281.
E-mail address: miller@chem.fsu.edu (B.G. Miller).

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of this communication network. Perhaps the most frequently encountered form of cooperativity can be found in the thermodynamics of ligand binding, where the prior association of a ligand alters the affinity of a subsequent binding event [1,2]. The association of oxygen with tetrameric hemoglobin serves as the prototypic example of thermodynamic cooperativity [3]. A different form of cooperativity can be observed in the kinetic response of allosteric enzymes, in which the association of a ligand at a site distinct from the active site impacts the rate of substrate transformation [4,5]. Aspartate carbamoyltransferase has emerged as a classic example of enzymatic allostery [6]. In the aforementioned cases, cooperativity involves multiple binding sites, often contained within discrete monomer units that assemble into higher order structures. It is important to note, however, that cooperativity can occur in the absence of multiple binding sites and without macromolecular oligomerization [7–11].

A less common manifestation of cooperativity occurs in the non-Michaelis–Menten kinetic response of certain monomeric enzymes with single ligand-binding sites. First theorized to exist more than 40 years ago [9,12], kinetic cooperativity in monomeric enzymes has been established in only a small number of cases. The best-studied example of this type of cooperativity is provided by human glucokinase, an enzyme that plays a key role in glucose homeostasis [13]. Glucokinase is a monomeric enzyme that contains a single binding site for glucose, yet displays a sigmoidal kinetic response to increasing glucose concentrations that is characteristic of positive cooperativity [14,15]. The physiological importance of this enzyme as the body's principle glucose sensor has resulted in a variety of structural and mechanistic studies over the last decade. We will refer to glucokinase throughout this review, as it has emerged as a model system for understanding kinetic cooperativity in monomeric enzymes.

The purpose of this mini-review is to provide an updated picture of kinetic cooperativity in monomeric enzymes for the unfamiliar reader. Rather than providing a detailed description of the kinetic equations developed to treat monomeric cooperativity, for which several excellent references already exist [16–18], we will briefly highlight the principle mechanisms that are postulated to give rise to this phenomenon. We then turn our attention to recent experimental approaches that may offer new insights into the nature of slow conformational transitions associated with monomeric cooperativity. Finally, we conclude with a brief discussion of the physiological relevance of cooperativity in monomeric enzymes with single ligand-binding sites.

1.1. Classifying and quantifying cooperativity

Cooperativity can impact either the thermodynamic or kinetic properties of a biological system. Thermodynamic effects of cooperativity are usually observed as an alteration in ligand binding affinity resulting from the presence of an additional ligand that occupies a structurally distinct site [1,2]. Kinetic cooperativity, in contrast, is observed as a deviation from hyperbolic kinetics in the steady-state response of an enzymatic process [4]. Cooperativity in monomeric enzymes with single ligand-binding sites is exclusively kinetic in origin. As a result, the Michaelis–Menten equation is insufficient to describe the dependence of the rate upon varying substrate concentration. Thus, new mathematical treatments are required to describe the rates of monomeric single-site enzymes that display cooperativity.

In principle, kinetic cooperativity in monomeric enzymes can be represented by appropriate modifications of several well-known models originally developed to treat equilibrium cooperativity in oligomeric systems. However, the most commonly employed treatment utilizes a modified version of the Michaelis–Menten equation based upon the formulation first developed by Archibald Hill [19] (see Scheme 1).

In this expression, the extent of cooperativity is reflected by the value of the Hill coefficient, n. When the value of $n = 1$, the system is classified as non-cooperative and the Hill formalism simplifies to the standard Michaelis–Menten equation. When $n < 1$, the system is negatively cooperative (substrate association reduces the reaction velocity) and when $n > 1$, the system is positively cooperative (bound substrate enhances the reaction velocity). Cooperativity is most easily visualized by performing a simple logarithmic transformation of the Hill expression. In the equation shown in Scheme 2, the value of the slope, n, readily depicts the positive or negative nature of the cooperative effect upon the reaction rate [20]. If the Hill formalism is used to describe systems that display thermodynamic cooperativity, as is the case when oxygen associates with hemoglobin, n has a maximum value that is equal to the number of ligand-binding sites present in the macromolecular assembly [19,21]. No such equivalency is possible for a cooperative monomeric enzyme with a single ligand-binding site, thus the Hill coefficient has no operational meaning in these instances. It simply serves as a convenient measure of the relative magnitude of the cooperative effect.

1.2. Kinetic hysteresis and monomeric cooperativity

Enzymes that display a slow response to alterations in ligand concentration have been termed hysteretic [22]. In many instances, the hysteric response can be attributed to slow transitions in enzyme structure that accompany substrate binding or product release. While hysteresis produces a time-dependent alteration in enzyme activity, it does not necessarily result in kinetic cooperativity. The manifestation of hysteresis can be observed as a burst or lag in substrate utilization without the generation of cooperative steady-state kinetics. The observation of hysteresis depends upon the availability of an experimental setup that affords direct observation of enzyme activity over the time window during which the transitions occur. Kinetic cooperativity resulting from hysteresis is most commonly observed in the steady-state response of an enzyme's reaction rate across a range of substrate concentrations. The production of kinetic cooperativity in monomeric enzymes generally requires that the hysteretic transitions take place at a rate comparable to catalytic turnover, that substrate binding does not reach equilibrium, and that alterations in substrate concentration shift the enzymatic reaction through distinct catalytic cycles [23]. A more detailed description of the relationship between hysteresis and kinetic cooperativity can be found in Frieden's treatment of this topic [24].

2. Mechanisms of monomeric kinetic cooperativity

Similar to cooperativity in systems containing multiple ligand binding sites, cooperativity in monomeric single-site enzymes is often linked to protein conformational changes. Indeed, in most cases investigated to date, monomeric cooperativity has been attributed to slow, substrate-induced alterations in enzyme structure that prevent substrate binding from reaching equilibrium on the timescale of catalytic turnover [4]. Below we describe the models most commonly enlisted to explain kinetic cooperativity in monomeric enzymes. Interested readers are referred to more detailed treatments of each model, which are available in the primary literature [16,18].

2.1. Mnemonic model

Ricard, Meunier and Buc first formulated the mnemonic model in an effort to explain the ability of monomeric enzymes to display

$$v = \frac{V_{max}[S]^n}{K_{0.5}^n + [S]^n}$$

Scheme 1. The Hill equation is used to quantify the degree of cooperativity displayed by an enzyme [19].

$$\log\left(\frac{v}{V_{max} - v}\right) = n\log[S] - n\log K_{0.5}$$

Scheme 2. Logarithmic transformation of the Hill equation, where the value of the slope, n, reveals the type of cooperativity.

non-hyperbolic kinetics [16,25]. Their model expanded on earlier conceptual work by Rabin and Frieden who postulated that the conformation of an enzyme following product release could be different from the initial enzyme state [9,22]. The name for the mnemonic model was borrowed from Whitehead who suggested that an enzyme has the ability to "remember" a substrate-induced conformation for a short time after the catalytic cycle is complete [26]. As put forth by Ricard, Meunier and Buc, the mnemonic model involves the oscillation between two distinct enzyme species – a low-affinity state (E*) and a high-affinity state (E) (Fig. 1A). To produce positive cooperativity, the equilibrium strongly favors the low-affinity conformation in the absence of substrate. The catalytic cycle begins when substrate binds to the enzyme and induces a conformational transition to a new ligand bound state (E′S). Depending upon the molecularity of the enzymatic reaction, catalysis can occur in the E′S state, or a second substrate can associate prior to substrate transformation. After the chemical reaction, product(s) is released to regenerate the high-affinity state (E). If substrate is abundant, the high-affinity state can rapidly bind another molecule of substrate and undergo a second round of catalysis without formation of the slowly realized, low-affinity conformation. However, if substrate concentrations are low, the enzyme slowly relaxes to the low-affinity conformation before another molecule of substrate has time to associate with the enzyme. The failure of substrate binding to equilibrate with the conformational ensemble due to the slow rate of conformational interconversion is responsible for generating a non-Michaelis–Menten kinetic response.

2.2. Ligand-induced slow transition model

A second model for kinetic cooperativity in monomeric enzymes is the ligand-induced slow transition (LIST) model, which

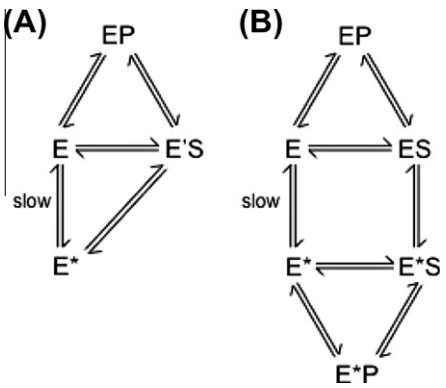

Fig. 1. Two proposed mechanisms for cooperativity in monomeric enzymes with single ligand-binding sites. Both the mnemonic model (A) and the ligand-induced slow transition model (B) require the slow interconversion of two enzyme conformations, a low-affinity state (E*) and a high-affinity state (E). In the original mnemonic proposal [14], the collision of substrate (S) with either form of the enzyme induces a new conformational state (E′).

was developed by Neet et al. [18] and Cardenas et al. [27]. The LIST model provides a more general mechanism to explain the basis for kinetic cooperativity in a monomeric enzyme. Similar to the mnemonic model, the LIST mechanism involves the existence of two distinct enzyme conformations, E* and E. In the LIST model, however, a pre-existing equilibrium exists between the two enzyme species in the absence of substrate (Fig. 1B). These two conformations possess different affinities for substrate, and the equilibrium between these states is controlled by the substrate concentration. Similar to the mnemonic mechanism, the interconversion between enzyme conformations must occur more slowly than turnover, thus preventing equilibration during substrate association. The postulated slow transition implied by this model can be an isomerization event or an association–dissociation process. The ligand-induced slow transition model postulates that each conformation has the ability to undergo its own catalytic cycle, and the resultant steady-state velocity is the sum of the rates for the two catalytic cycles. An interesting corollary of the LIST model is that chemical transformation of the substrate proceeds through multiple transition states, each of which are associated with a conformationally distinct enzyme species. Similar concepts have been debated in evaluating the ability of induced fit conformational changes to provide specificity in enzymatic reactions [28,29].

2.3. Random addition of substrates in bimolecular reactions

A conceptually distinct mechanism for monomeric cooperativity has been postulated for enzymes that catalyze bisubstrate reactions. This model was first delineated by Ferdinand [7] and further developed by Pettersson [30] in an attempt to reconcile the sigmoidal kinetic response of mammalian liver glucokinase. It requires that the enzymatic reaction be capable of proceeding through a random order mechanism. Unlike the more commonly encountered mnemonic and LIST mechanisms, the random order model does not rely upon enzyme conformational heterogeneity or slow interconversion rates. According to this model, random addition of substrates can produce cooperative behavior when one particular pathway of substrate addition is kinetically preferred. Although the kinetically disfavored pathway contributes negligibly to the steady-state reaction velocity, its existence provides a mechanism by which a "non-productive" intermediate ES binary complex can accumulate. Given proper substrate concentrations and appropriate ratios of microscopic rate constants, such a condition can give rise to substantial deviations for Michaelis–Menten kinetics. Although this mechanism has received only modest attention in the biochemical literature, at least one enzyme, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase from *Rhodomicrobium vannielli* appears to function according to this type of mechanism [31].

3. Types of "slow" conformational change

A critical component of both the mnemonic and LIST mechanisms of kinetic cooperativity is the existence of slow conformational changes that prevent substrate binding from reaching equilibrium. To confer cooperativity to a monomeric enzyme, conformational changes must occur with a rate constant comparable to, or slower than, substrate turnover [4]. Assuming that most enzymes are characterized by k_{cat} values that span the range of 1–5000 s^{-1}, the conformational changes responsible for cooperativity must have half-lives slower than 1 ms. In this section, we take a moment to discuss the types of conformational changes that operate within the millisecond time regime. These movements, which occur at a rate similar to or slower than catalysis, have been

termed tier-0 dynamics [32]. In some instances, they appear to represent the rate-limiting steps of substrate transformation.

3.1. Proline cis–trans isomerization

Proline is the only natural amino acid with the ability to form a stable *cis* peptide backbone conformation [33]. Depending upon the identity of the preceding amino acid, the *cis* to *trans* equilibrium constant in prolyl peptide bonds can range from 0.05 to 1 [33–35]. The ability to sample both *cis* and *trans* conformations often has a dramatic structural impact upon the polypeptide. The *trans* to *cis* interconversion causes a compaction of the surrounding polypeptide chain, which can propagate throughout the protein thereby facilitating communication between distant sites. An example where sampling both *cis* and *trans* prolyl bond conformations affords access to discrete structures is provided by the interleukin-2 tyrosine kinase, Itk [36]. The SH2 domain of Itk undergoes a large-scale conformational switch resulting from proline isomerization. The resulting states differ in structure and function, with distinct conformations of the enzyme recognizing distinct substrates.

In addition to providing access to distinct structures, proline *cis*/*trans* isomerization is characterized by a substantial energetic barrier. Kinetic measurements demonstrate that prolyl peptide bond isomerization can occur with rate constants as slow as 0.002 s^{-1} [37]. Indeed, prolyl *cis*/*trans* isomerization has been shown to function as a molecular timer that dictates the rates of many physiologically important polypeptide conformational changes [38]. The biological consequence of slow prolyl bond isomerization is emphasized by the existence of enzymes with the specific purpose of accelerating this process [38,39]. To date, monomeric kinetic cooperativity has not been shown to involve prolyl isomerization, nevertheless the sluggish time scale of this conformational reorganization indicates that such a case is possible.

3.2. Lid motions

Loops are perhaps the most mobile components of protein structure. Enzyme active sites are sometimes covered by flexible loops capable of sampling distinct conformational states depending upon the presence or absence of a ligand. In these instances, an open loop conformation facilitates substrate binding and product release, whereas a closed loop envelops the substrate and excludes bulk solvent from the active site. Oftentimes loop closures involve rigid-body movement of the loop about two hinge regions. The internal conformation of the loop does not change significantly and the loop simply functions as a solid lid for the ligand-binding site. Kinetic investigations have established that lid motions can occur within the microsecond to millisecond time regime and can correlate with enzymatic turnover rates [40,41]. In several cases conformational reorganizations of active site loops are partially or fully rate determining, demonstrating that lid motions could contribute to the generation of cooperativity in monomeric systems [42].

Two examples of enzymes in which lid motions have been shown to represent a slow step in the catalytic cycle are triosephosphate isomerase (TIM) and adenylate kinase (Adk). TIM catalyzes a simple proton transfer reaction that results in the interconversion of dihydroxyacetone-3-phosphate and glyceraldehyde-3-phosphate. The enzyme adopts a $(\beta/\alpha)_8$-barrel fold and contains a single flexible loop consisting of 11 amino acids [43]. This loop adopts at least two distinct structures, one of which is observed in the apo enzyme structure and another when substrate analogs are present at the active site [44]. Solid-state NMR experiments and laser-induced temperature jump relaxation spectroscopy have demonstrated that product release is kinetically limited by the rate of loop opening in TIM [45,46]. A similar lid motion has been described in Adk, an enzyme that catalyzes the reversible conversion of ATP and AMP into two ADP molecules [47]. Adk contains a nucleotide-binding lid that sequesters the reacting substrates within the active site. A comparison of protein dynamics and catalytic activities of a mesophilic and a thermophilic Adk conducted at different temperatures suggests that opening of the nucleotide binding lid is rate-limiting for both enzymes. Moreover, the reduced catalytic activity observed when the hyperthermophilic enzyme is assayed at ambient temperatures, was attributable to a slower-lid opening rate. Although neither TIM nor Adk display kinetic cooperativity, these experiments demonstrate that lid motions can occur on the same time scale as turnover, thus raising the possibility that such motions could be slow enough to generate cooperativity in single-site monomeric catalysts.

3.3. Hinge-bending motions

Hinge-bending motions involve the rigid-body movement of two protein domains toward one another. The motion occurs in response to the association of a ligand at an internal binding site located along the interfacial region of both domains. Similar to other protein motions, hinge movements result in closure of the active site cleft providing an appropriate microenvironment for catalysis to occur. Multi-substrate enzymes also use hinge-bending motions to align substrates in the correct orientation with respect to one another [48]. Among the best-characterized examples of hinge-bending motions are those found within the ATP-dependent sugar kinases of central metabolism. One such example is human glucokinase, which represents the most thoroughly investigated example of a cooperative kinetic enzyme. Glucokinase displays a cooperative sigmoidal kinetic response to increasing glucose concentrations, but shows standard Michaelis–Menten kinetics when the second substrate, MgATP, is varied [4,13]. Kamata and coworkers successfully determined the 2.3 Å resolution crystal structure of a truncated form of human liver glucokinase in complex with glucose and a synthetic activator [49]. These investigators also determined the structure of unliganded glucokinase, albeit at a much lower resolution of 3.4 Å. The results of these studies revealed a dramatic change in enzyme conformation that occurs upon glucose association. When glucose binds to the human enzyme, the smaller C-terminal domain undergoes a 99° rigid body rotation toward the N-terminal domain, which remains largely stationary (Fig. 2). As a result of this hinge motion, the enzyme adopts a more compact structure. The hinge motion also appears to facilitate the sequestration of the α13-helix in the glucose bound state. In human glucokinase, as well as other non-cooperative catalysts, there is a notable lack of information regarding the rate constants of hinge-bending motions. Nevertheless, the hinge-motion observed in the cooperative transition of glucokinase suggests that these conformational rearrangements can be slow in comparison to turnover.

4. Methods to investigate slow conformational changes

A complete mechanistic understanding of kinetic cooperativity in monomeric enzymes requires both a kinetic and structural description of the conformational changes associated with substrate association. Unfortunately, this information has been slow to emerge, even for the best characterized examples of monomeric cooperativity. In the first few decades following the identification of cooperative monomeric enzymes, experimental work was largely limited to kinetic investigations using standard approaches. Unfortunately, these methods provided little structural insights. X-ray crystal structures exists for a few cooperative monomeric en-

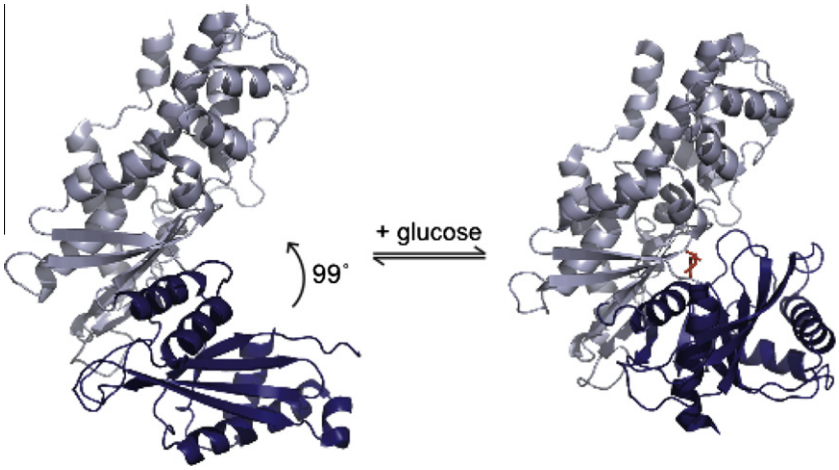

Fig. 2. The cooperative hinge-bending motion observed in human glucokinase. Upon glucose association, the smaller C-terminal domain (dark) undergoes a 99° rigid body rotation toward the N-terminal domain (light), which remains largely stationary.

zymes [49], and this data has assisted investigators in identifying the extent of conformational changes associated with cooperativity; however, because these structures are static, correlating structural changes with solution phase protein dynamics is difficult. It is clear that more detailed descriptions of monomeric cooperativity will require experimental methods that probe atomic level dynamics. In this regard, the advent of several new techniques offers much promise in elucidating the structural aspects of monomeric cooperativity. Two such methods are described below.

4.1. High resolution NMR

The development of multidimensional pulse sequences that probe magnetization exchange and relaxation processes has resulted in the widespread application of high-resolution NMR to the investigation of protein dynamics [50]. Combined with the advent of methods for the site-specific incorporation of various NMR active nuclei into the backbone and side chain atoms of proteins, these approaches enable one to probe conformational changes across a variety of time scales in proteins of varying sizes. Relaxation methods that probe the millisecond to second time regime are most important in measuring the rates of slow conformational changes. These include ZZ-exchange spectroscopy, which can measure exchange processes that occur with rate constants of 1–1000 s^{-1}, and CPMG relaxation methods, which are generally applied to systems with half-lives for exchange not less than 100 μs [51]. ZZ-exchange spectroscopy is accomplished by monitoring the exchange of longitudinal magnetization between two states. Exchange is visualized by the appearance of a cross peak between the individual signals originating from the exchanging states. Rates of exchange are quantified by the variation in cross-peak intensity observed over a variety of mixing times. CPMG relaxation spectroscopy monitors the relaxation of transverse magnetization in a two-state dynamic process. It affords information about the interconversion rate, the relative population of the exchanging species and the chemical shift difference between the two states. Both ZZ-exchange and CPMG relaxation methods have been applied to enzymes that do not display cooperativity in an effort to correlate protein dynamics with catalytic function [52–56]. The application of such methods to cooperative monomeric enzymes promises to provide the first direct measurement of slow conformational exchange in these systems.

In addition to the aforementioned relaxation methods, which provide dynamic information at a residue specific level, NMR can also be used to probe conformational changes more globally. One method to do so uses NMR to monitor the exchange of amide protons with a deuterated solvent [50]. This approach is widely amenable to a variety of target proteins, since it does not require site-specific labeling and instead relies upon the universal incorporation of ^{15}N into the protein. The rate of H/D exchange depends upon several factors including the extent of secondary and tertiary structure within the protein and the relative solvent accessibility of the protein backbone. In addition, the rate of H/D exchange is expected to depend upon the rate and magnitude of conformational transitions. This method was originally limited to small proteins, but improvements have pushed the size limit upward, and it now has the ability to analyze complexes as large as 100 kDa [57]. An example of the application of this method is provided by the work of Larion et al. who probed the conformational dynamics of human glucokinase using H/D exchange measurements [58]. In these studies, the investigators used amide proton exchange on universally ^{15}N-labeled preparations of glucokinase to demonstrate that the enzyme is highly dynamic and samples multiple conformational states, both in the absence and presence of glucose.

4.2. Single-molecule techniques

The ability to image single protein molecules in real-time under physiologically relevant conditions could make a significant contribution to our mechanistic understanding of cooperativity in monomeric single-site enzymes. Single-molecule methods can detect and characterize a variety of phenomena including molecular heterogeneity, transient intermediates and rare conformational events that are often hidden by ensemble averaging [59]. A range of single-molecule applications have been developed, including electrophysiology, force measurement and micromanipulation; however the pairing of fluorescence spectroscopy with single-molecule methods has proven most powerful in elucidating conformational heterogeneity of protein complexes. Single-molecule fluorescence experiments typically require modification of the protein with one or more highly sensitive fluorescent tags. These fluorophores can report on the relative orientation and distances sampled by the protein at the site of their attachment. The single-molecule fluorescence methods most frequently employed to monitor conformational changes in polypeptides include single-pair Förster resonance energy transfer (FRET) and fluorescence polarization anisotropy [60]. Combinations of these two methodologies are likely to provide a more complete description of the energy landscape sampled by cooperative monomeric enzymes. Each is briefly described below.

Single-pair FRET has the ability to measure distance changes between two sites on a macromolecule in a time-dependent fashion. FRET efficiencies can provide a measure of the amplitude of motion, and efficiency distributions can provide evidence for the sampling of multiple states [59]. Since only one donor–acceptor system is observed at a time, dynamic distance changes on a millisecond time scale can be monitored [32,60]. Importantly, the distance changes measured by single-pair FRET take place in solution, in real time. The interpretation of FRET data is often bolstered by corroborative X-ray crystallographic or NMR data [47]. One advantage of this single-molecule method is that it can easily be applied to very large macromolecular systems, and it can be used to investigate intramolecular reorganizations or intermolecular interactions. One such application is demonstrated by studies of F_0F_1-ATP synthase, an enzyme that uses an electrochemical proton gradient to drive ATP synthesis [61]. Single-pair FRET was used to observe the movement of various subunits during proton-powered ATP synthesis. Three distinct donor–acceptor fluorescence intensity ratio levels were observed, which were interpreted in terms of three distinguishable orientations of the enzyme during the sequence of motions associated with ATP synthesis and hydrolysis.

Single-molecule fluorescence polarization anisotropy (smFPA) reports on dynamic changes in the dipole orientation of an attached probe, which can be interpreted on the basis of the overall angular motion of the macromolecule [62]. smFPA measurements are based upon the use of polarized light to selectively excite fluorophores whose absorption transition dipoles are oriented parallel to the excitation photons. The subsequent fluorescence emission is also partially polarized. Molecular motion, however, reduces the fraction of fluorophores that emit in the direction of the incident light. The resulting alterations in anisotropy are often interpreted in the form of dynamic conformational changes of the molecule. The usefulness of this method in measuring protein conformational dynamics has been demonstrated with fluorescently labeled staphylococcal nuclease during the process of ligand binding [63]. The fluorescent probe, tetramethylrhodamine (TMR), showed rapid and unrestricted movement when attached to ligand-free staphylococcal nuclease. In the presence of the active site inhibitor, deoxythymidine diphosphate, TMR displayed hindered rotational dynamics and temporal fluctuations. The observed changes in smFPA reflect changes in protein dynamics resulting from inhibitor binding. Notably, smFPA can be monitored in a time-dependent fashion over a period ranging from milliseconds to seconds. Thus, this experimental technique offers the potential to directly observe conformational changes in the time regime that is expected to result in monomeric kinetic cooperativity.

5. Monomeric single-site enzymes suspected of displaying kinetic cooperativity

For many years cooperativity was thought to require the presence of multiple ligand-binding sites in oligomeric systems. The earliest experimental evidence of non-Michaelis–Menten kinetics in a monomeric enzyme with a single ligand-binding site appeared in the late 1960s [14]. In the subsequent decade, several additional cases of this curious kinetic phenomenon were observed. Nevertheless, monomeric cooperativity is still far from commonplace. Ricard suggested that the scarcity of this phenomenon was due to the fact that cooperativity in a monomeric enzyme could only be achieved at the expense of catalytic efficiency [64]. Another potential reason for the dearth of cooperative monomeric enzymes is the difficulty with which enzyme reaction rate data can be accurately determined at very dilute substrate concentrations, where positive cooperativity is expected to be most noticeable. No matter the reason, the number of enzymes that appear to represent real cases of monomeric kinetic cooperativity is small. A list of these enzymes is provided in Table 1.[1]

Table 1
Cooperative monomeric enzymes with single ligand-binding sites.

Enzyme (Ref.)	Source	Cooperativity (substrate)
JH glucosidase I [65]	*Apis cerana japonica*	Positive (turanose)
		Negative (p-nitrophenyl α-glucoside, sucrose, maltose)
WH glucosidase I [65]	*Apis mellifera*	Positive (turanose, maltodextrin)
		Negative (phenyl α-glucoside, p-nitrophenyl α-glucoside, sucrose, maltose)
δ-Chymotrypsin [66]	Human	Negative (ester substrates)
Acid phosphatase [67]	Maize	Negative (p-nitrophenyl-phosphate)
Cytochrome P450 monooxygenase 3A4 [68–74]	Human	Positive (various substrates)
		Negative (BRL 3287, naphthalene)
Hexokinase I [75,76]	Human	Negative (G-6-P)
Hexokinase II [77]	Human, rat	Negative (glucose, ATP, G-6-P)
Hexokinase III [78]	Human, rat	Positive (glucose)
Glucokinase [15,79–81]	Human, rat	Positive (glucose)
Octopine dehydrogenase [82]	*Pecten maximus* L.	Negative (NAD^+)
Ribonuclease I [83–86]	Rat	Positive (various substrates)
Ribonucleotide triphosphate reductase [87,88]	*L. leischmannii*	Positive (ribonucleotides)
Epoxide hydrolase 1 [89,90]	*Solanum tuberosum*	Positive (various epoxides)
Thymidine kinase [91–94]	Human	Negative (ATP, deoxythymidine)

6. Conclusion

Several of the monomeric catalysts shown in Table 1 are known to play key roles in cellular metabolism. As such, these enzymes represent logical sites for regulation. It is likely that the existence of cooperativity in monomeric enzymes is not simply an incidental consequence of the inherent flexibility of proteins. Instead, kinetic cooperativity appears to provide organisms with the advantageous ability to slowly respond to changes in metabolic flux [22–24]. It is a difficult task, however, to definitively establish the physiological importance of cooperativity. Ultimately this requires the direct observation of the results of cooperativity *in vivo*, as well as the corresponding consequences of its absence. In this regard, the most convincing evidence for the biological importance of kinetic cooperativity can be found in genetic and biochemical investigations of human glucokinase.

The sigmoidal kinetic response of glucokinase is characterized by a $K_{0.5}$ value of 8 mM, which is near the mean blood glucose levels in humans [95]. As a result, the cooperative response of glucokinase allows the enzyme to be most responsive across a range of glucose concentrations that are biologically relevant. The importance of this regulatory mechanism is evidenced by two disease states. Genetic lesions in the *glk* gene, which reduce the activity of the enzyme below that of wild-type, result in maturity onset diabetes of the young (MODY) [96]. Conversely, patients suffering from persistent hyperinsulinemia of infancy (PHHI) possess a variant form of glucokinase that is hyperactive [97]. In glucokinase variants associated with PHHI, the glucose kinetic response curve is shifted to a lower $K_{0.5}$ value and cooperativity is dramatically re-

[1] It is important to note that some enzymes listed in Table 1 may be subject to experimental artifacts, such as substrate inhibition or the presence of isozymes during enzyme assays, which are known to cause the appearance of cooperativity [4].

duced. This results in oversecretion of insulin under hypoglycemic conditions, which, in severe cases, can be lethal. Together, MODY and PHHI emphasize the physiological importance of setting the $K_{0.5}$ value of glucokinase at the intermediate level of 5 mM. Indeed, the loss of kinetic cooperativity in this enzyme impacts glucose homeostasis in the entire body. Although the glucokinase case study may represent an extreme example of the importance of cooperativity in monomeric, single ligand-binding site enzymes, it also accentuates the importance of this phenomenon. Continued searches for examples of this unique kinetic characteristic are likely to uncover new regulatory features in important cellular systems.

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