Current Biology 16, 2150–2155, November 7, 2006 ©2006 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2006.09.013

Report

An Entropic Mechanism to Generate Highly Cooperative and Specific Binding from Protein Phosphorylations

Peter Lenz[1] and Peter S. Swain[2,*]
[1] Fachbereich Physik der Philipps-Universität
Renthof 5
D-35032 Marburg
Germany
[2] Centre for Nonlinear Dynamics
Department of Physiology
McGill University
3655 Promenade Sir William Osler
Montreal, Quebec H3G 1Y6
Canada

Summary

Cooperative interactions are essential to the operation of many biochemical networks. Such networks then respond ultrasensitively in a nonlinear manner to linear changes in network input, and network output, for example, levels of a phosphorylated protein or of gene expression, becomes a sigmoidal function of concentrations of input molecules. We present a novel, entropic ultrasensitivity mechanism that generates highly cooperative and specific binding between two proteins. We consider a disordered protein with multiple phosphorylation sites that binds to a single binding site on an interacting protein. We assume that each phosphorylation locally orders the protein. Such local order affects protein conformational entropy nonlinearly and generates binding that is a highly cooperative function of the number of protein phosphorylations (with Hill coefficients well above 10). Substantial binding may only occur once the disordered protein is phosphorylated a critical number of times or more. Cooperativity is determined by the size of the disordered region of the protein, the binding affinity, and unusually the concentration of the interacting protein. Given the widespread occurrence of disordered, multiply phosphorylated proteins, its highly ultrasensitive character, and the ease of its control, entropic, phosphorylation-driven cooperativity may be extensively exploited intracellularly.

Results

Consider a disordered protein A that interacts with a single binding site on protein B to form a complex C, i.e., $A + B \leftrightharpoons C$ (Figure 1). We use equilibrium thermodynamics to describe their interaction. Any chemical reaction equilibrates when the Gibbs free energy

$$G = E + pV - TS \quad (1)$$

comes to a minimum. The reactants and products involved have total energy E and entropy S, and the reaction occurs at temperature T, pressure p, and volume V. For reactions occurring in the cell, and so in solution, there is little change of volume and pressure. We will therefore neglect the pV term in G: It contributes only a constant.

Both energy and entropy determine the equilibrium of a reaction. The average kinetic energy per molecule is set by temperature and is fixed. The negative interaction energy between A and B once bound in complex C favors binding and lowers G. Nevertheless, the reaction does not proceed to form as much C as possible. The number of molecules decreases each time a complex forms, and this reduction reduces system entropy. Equilibrium is only reached once the entropy reduction matches the increase in negative binding energy (see the Supplemental Data available with this article online).

There are four contributions to the entropy of interaction: translational, rotational, and conformational entropies and the entropy of solvation [1]. The translational entropy per molecule varies inversely with the logarithm of concentration, but the total translational entropy is proportional to the number of molecules. It disfavors a reaction that decreases the total molecule number. The conformational entropy of a molecule is determined by the number of different structural conformations of the molecule. For the hard-sphere model of molecules, typically considered in calculations of reaction free energies, just one conformation is available to each molecule, and the conformational entropy is zero.

Conformational entropies are usually ignored, but we consider the reactant A to be an intrinsically disordered protein. It has been proposed that proteins exist in four different structural states: an unstructured (completely disordered) state, a molten globule state (having secondary but no tertiary structure), a beads-on-string state (in which folded domains are connected by flexible, extended, and unstructured linker regions), and a mostly folded state (only local disorder) [2]. An unstructured protein will sample many different structural conformations in solution; its conformational entropy is unlikely to be negligible.

Once bound to B, we assume that the number of conformations available to A is drastically reduced. Many disordered proteins fold or at least form some structure once they are bound to their target proteins [2]. Although we do not claim that A must completely fold when bound to B, we do assume that the number of conformations available to A once it is bound is negligible in comparison to the number of conformations available to free A (Figure 1).

Including translational entropy and the conformational entropy of free A, the fraction of A molecules bound to B at equilibrium, ρ, can be calculated (see Supplemental Data):

$$\rho = \frac{1}{1 + K_{\text{eff}} \exp(S^{\text{conf}}/k_B)} \quad (2)$$

*Correspondence: swain@cnd.mcgill.ca

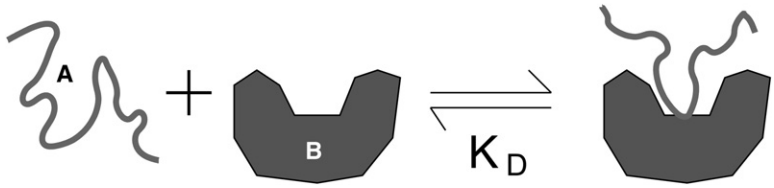

Figure 1. Binding of a Disordered Protein *A* to a Single Site on Protein *B*

An equilibrium exists between free and bound protein. Protein *A* is relatively restricted in its movements when bound to *B* but can fluctuate freely in solution.

where S^{conf} is the conformational entropy of a free *A* molecule, k_B is Boltzmann's constant, and K_{eff} obeys

$$K_{eff} = K_D/[\mathrm{B}] \quad (3)$$

for an equilibrium *B* concentration of [B]. In Equation 3, K_D is the dissociation constant of the complex *C* in the absence of any conformational entropy change between free and bound *A*. If S^{conf} is zero, *A* is then ordered both in solution and bound to *B*. Equation 2 recovers standard mass action: As K_{eff} decreases, the fraction of bound *A* increases hyperbolically in a Michaelis-Menten-like manner. For large S^{conf}, however, ρ is small. Although there is a gain in (negative) binding energy when *A* and *B* form *C*, the reaction is penalized by the resulting loss in conformational entropy of *A*, and the conformational entropy loss is too great to be offset by the gain in binding energy.

We assume that *A* can be phosphorylated several times and that each phosphorylation imposes a local structural order and reduces *A*'s conformational entropy. No assumption is made about *A*'s binding site for *B*; the binding site could always be available or could be induced by phosphorylation. Figure 2A shows a typical conformation for a 2D model of a disordered protein. For simplicity, we assume for Figure 2 that the protein consists of 20 "units" of equal size, each connected by flexible linkers. These units could be entire protein domains as in the beads-on-string model or small groups of amino acids that maintain their relative orientation as the protein fluctuates. The precise details are not important for our argument. Figure 2B shows the same protein phosphorylated six times, each time on a linker domain. We assume that each phosphorylation "kinks" the protein, with the flexible linker domain adopting a more rigid conformation (here a right angle). This phosphorylation-induced kinking dramatically reduces the number of conformations available to the protein. Figures 2C and 2D show how the cloud of possible protein conformations reduces in size once the protein has been phosphorylated. Such a reduction in the number of protein conformations gives a corresponding reduction in the protein's conformational entropy.

The number of *A* conformations in fact decreases nonlinearly with an increase in the number of phosphorylation-induced kinks (Figure 3, right-hand axis and dotted curve). Two protein units cannot occupy the same physical space, and the number of conformations that have this self-avoidance property reduces significantly with an increase in protein phosphorylations. The conformational entropy of a single protein, S^{conf}, is defined as [3]:

$$S^{conf} = k_B \log(\text{number of protein conformations}). \quad (4)$$

Via Equation 2, a nonlinear decrease in the number of protein conformations can lead to a highly sigmoidal change in the fraction of bound *A* as a function of its phosphorylation state (Figure 3, left-hand axis).

Entropic, phosphorylation-driven binding can be highly sigmoidal (Figures 3 and 4). The degree of a sigmoidal response is often characterized by a Hill coefficient: the exponent *n* in the sigmoidal Hill function. In our case, the Hill function is parameterized by $x^n/(m_{50}^n + x^n)$ where *x* denotes the number of phosphorylated sites and m_{50} is the number of phosphorylations required for 50% of the *A* protein present to be bound to *C*. Entropic, phosphorylation-driven binding often gives Hill coefficients well above 10 and sometimes as high as 33 (Figure 3). These numbers should be compared to the Hill coefficient of hemoglobin (about 3) [4] or to the Hill coefficient describing binding of the p53 protein to DNA (about 2) [5]. Although our predictions are for an idealized model, they do show the potential of entropic, phosphorylation-driven binding to generate highly cooperative behavior.

Our model predicts the cooperativity of binding increases with K_{eff} and with the length of the disordered protein. Binding is dominated by a low K_{eff}, corresponding to a high concentration of *B* or to a large binding

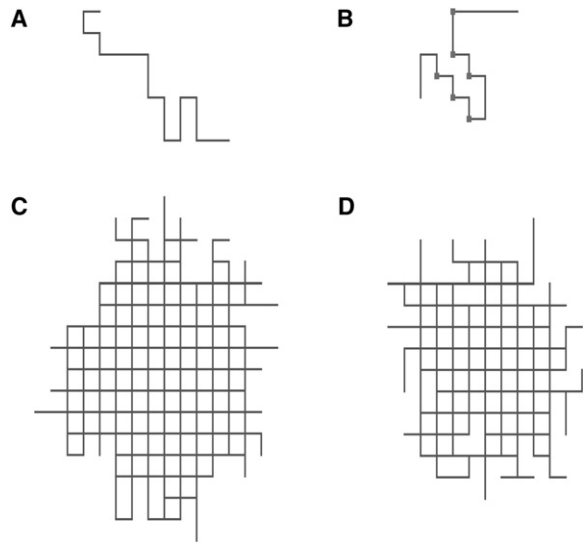

Figure 2. Examples of Conformations of an Idealized Disordered *A* Protein

(A) A typical conformation of an unphosphorylated protein of 20 monomers.

(B) A conformation for the same protein, now with six phosphorylations (marked by dots). Moving from the center of the protein, each phosphorylation forces a clockwise 90° "kink."

(C) A superposition of 100 attempts to randomly generate a conformation of an unphosphorylated protein (as in [A]). Some attempts are rejected because they are unphysical: The protein intersects itself.

(D) A superposition of 100 attempts to generate conformations for the same protein phosphorylated six times (as in [B]).

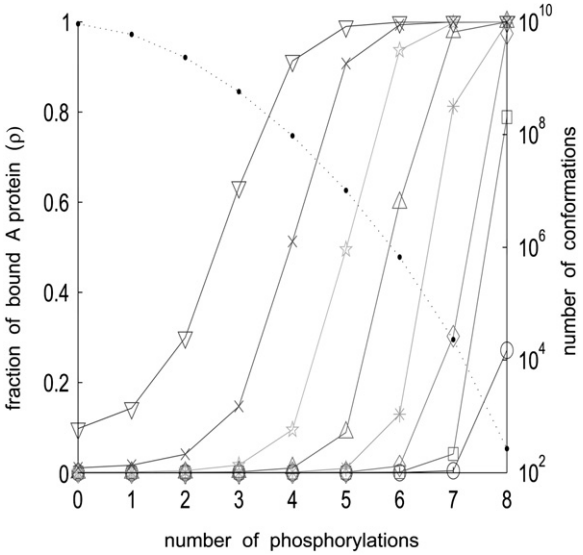

Figure 3. Example Binding Curves for Different Values of K_{eff} as a Function of the Number of Phosphorylations of the Disordered *A* Protein

The protein has 14 equally sized units with eight phosphorylation sites at positions 1, 2, 3, 5, 7, 9, 11, and 12. The right-hand *y* axis and dotted curve shows the number of conformations (in log space) of free *A* (calculated for a self-avoiding walk in 3D). Although we assume that once bound to *B*, the number of conformations available to *A* is small, free and phosphorylated *A* can still be significantly disordered before binding. For example, free *A* with five phosphorylations can have approximately 10^7 conformations (right-hand *y* axis). From left to right, the value of K_{eff} for each curve increases by a factor of 10. For the upside-down triangles, $K_{eff} = 10^{-9}$; for the crosses, $K_{eff} = 10^{-8}$ (Hill coefficient of approximately 8 and m_{50} of 4); for the stars, $K_{eff} = 10^{-7}$ (Hill coefficient of 13 and m_{50} of 5); for the rightside-up triangles, $K_{eff} = 10^{-6}$ (Hill coefficient of 17 and m_{50} of 6); for the asterisks, $K_{eff} = 10^{-5}$ (Hill coefficient of 22 and m_{50} of 6.5); for the diamonds, $K_{eff} = 10^{-4}$ (Hill coefficient of 33 and m_{50} of 7); for the squares, $K_{eff} = 10^{-3}$; and for the circles, $K_{eff} = 10^{-2}$.

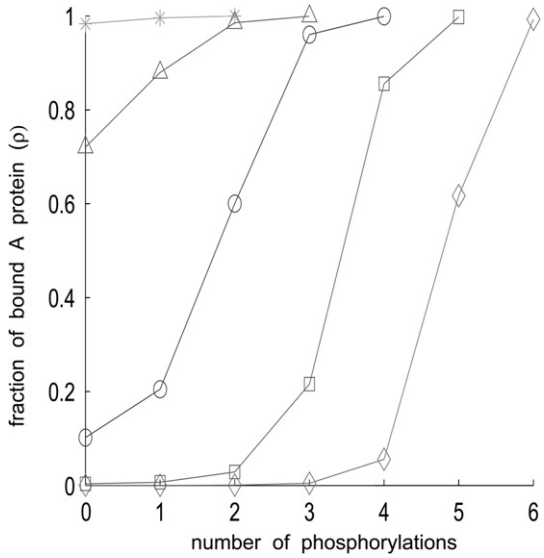

Figure 4. Typical Binding Curves for Idealized, Disordered Proteins of Different Lengths

Each alternate unit on each protein has a phosphorylation site. The binding affinity, K_{eff}, is 10^{-6}. Protein length increases from left to right. For the asterisks, the protein has six units and two phosphorylation sites, for the triangles, it has eight units and three phosphorylation sites; for the circles, it has ten units and four phosphorylation sites; for the squares, it has 12 units and five phosphorylation sites; and for the diamonds, it has 14 units and six phosphorylation sites.

energy between *A* and *B* (a small K_D). Only large changes in conformational entropy are then important, and a phosphorylation can only smoothly alter the binding curve (Figure 3). For small proteins, the relative importance of the conformational entropy decreases: the smaller the protein, the fewer its conformations (at least for idealized proteins consisting of units of equal length). Kinks in protein structure induced by phosphorylations contribute less to the free energy, and the binding curve is less sigmoidal (Figure 4).

Discussion

Cooperativity can occur allosterically [6, 7]; it can arise from the cyclic action of kinases and phosphatases [8], from cascades of enzymes [9], from protein multimerization [10] and translocation [11], from stoichiometric inhibitors [12], and from selective degradation mechanisms [13]. It is essential, for example, for biochemical systems to be able to switch irreversibly between different states, and such cooperativity is the basis for the transitions underlying development [14] or the establishment of memory [15].

Here, we have shown that phosphorylations can cause the binding of a disordered protein to its target protein to be highly cooperative. Our model has two assumptions:

(1) The binding protein is disordered when it is free. For simplicity, we assumed that the entire protein is disordered in our simulations, but it is more common that proteins in vivo are only partly disordered. Providing that the disorder still contributes to the conformational entropy of the protein, our argument holds. Once bound, we assume that the number of conformations available to the protein is negligible.

(2) Phosphorylations or, more generally, covalent modifications locally increase protein order. The protein need not become rigid at the site of the covalent modification, but the modification must reduce movement in some part of the protein.

We allow the binding domain of the disordered protein either to be always available for binding or to form upon protein phosphorylation.

There are a large number of proteins that satisfy our first assumption. Over 450 proteins are known to be partly or completely disordered (www.disprot.org), and 45% of eukaryotic proteins are predicted to have disordered regions of 30 or more consecutive amino acids, rising to 65% for proteins involved in cell signaling [16]. Indeed, most phosphorylation sites are predicted to be in disordered rather than ordered protein regions, particularly for serines and threonines [17].

Phosphorylations have long been known to change protein conformations [18]. Nuclear magnetic resonance (NMR) experiments show that phosphorylation on serines and threonines in peptides can change the structure

of the peptide backbone [19]. Unlike tyrosines, this effect is independent of the presence of charged groups in the surrounding sequence [19]. Molecular dynamics simulations also show that serines can undergo conformational changes upon phosphorylation [20]. We assume that phosphorylations locally increase protein order. There are many examples of such behavior: Serine phosphorylation of a peptide from tyrosine hydroxylase causes the peptide backbone to fold into a compact structure [21]; phosphorylation of a serine at the N terminus of an α helix can stabilize the helix [22]; phosphorylation of serines and threonines in the microtubule-binding protein tau induces proline-rich sequences to undergo a conformational change into a type II polyproline helix, a rigid and extended structure [23]; phosphorylation of a tyrosine reduces structural flexibility in the Shc adaptor protein [24]; and the SH2-SH3 connector region clamps these two domains upon the phosphorylation of a tyrosine in Src kinases [25].

Phosphorylation-induced prolyl isomerizations can also increase local protein order. Protein conformations can be changed by *cis-trans* interconversions of proline residues. This process alters local structure and can provide a "backbone switch." It is partly controlled by phosphorylation [26]. Ser-Pro and Thr-Pro motifs are common in signaling proteins. Depending on local structural constraints, phosphorylated motifs favor either a *cis* or *trans* configurations, but phosphorylation also slows spontaneous prolyl isomerization [26] and thus potentially stabilizes the particular configuration adopted. A phosphorylated motif is also a binding site for Pin1, a prolyl isomerase [27]. Phosphorylation of a Ser-Pro or Thr-Pro motif can therefore induce a conformation change by Pin1 recruitment, which, in turn, catalyzes prolyl isomerization. An example is the Cdc25C phosphatase, which controls mitotic initiation in vertebrates and has five Ser-Pro and Thr-Pro motifs—all of which are potential Pin1 substrates. The resulting conformational changes are significant enough to affect Cdc25C's catalytic activity [28].

Two types of binding have been proposed for multiply phosphorylated proteins [29]: a two-stage process where a few phosphorylations, that are essential for binding, are first required to prime the protein for further phosphorylation before binding occurs or an unordered process where each phosphorylation site increases the potential to bind and the number of phosphorylations determines binding, rather than the particular sites phosphorylated.

The second, unordered binding process has the characteristics of entropic, phosphorylation-driven binding. There are several examples: Gcn4, a transcriptional activator in yeast, has five serine and threonine phosphorylation sites. It is degraded via the SCF^{Cdc4} ubiquitin ligase, which has a single binding site for a phosphoepitope [30]. No significant change in ubiquitination occurs when each single phosphorylation site is mutated, but a marked reduction occurs for a mutation of all five sites [31]. When phosphorylated, the human hepatoma upregulated protein (HURP) is degraded by SCF^{Fbx7}. It has nine serine and threonine phosphorylation sites. Mutation to alanine of each individual site and some pairs of sites does not affect HURP binding to Fbx7, whereas mutation of all nine sites completely abolishes binding [32]. Rad9 is a yeast DNA-damage-checkpoint gene. Its protein has a domain with six serine and threonine phosphorylation sites and is phosphorylated to enable binding to Rad53. Single site mutations to alanine of these phosphorylation sites have little effect on Rad53 binding, and restoring single mutations in an all alanine mutant never fully recovers wild-type binding [33]. Finally, when phosphorylated, the yeast cyclin-dependent kinase inhibitor Sic1 is bound by SCF^{Cdc4} and ubiquitinated. Sic1 has nine serine and threonine phosphorylation sites, and phosphorylation on at least six is required for Cdc4 binding [34].

Our model requires these proteins to be disordered when unphosphorylated. Because experimental confirmation is not yet available, we used the PONDR tool to predict intrinsic disorder [35, 36]. Gcn4 is predicted to have a largest disordered domain (LDD) of 46 residues (and to be 60% disordered in total), HURP is predicted to have a LDD of 119 residues (55% disordered in total), Rad9 is predicted to have a LDD of 75 residues (45% disordered in total, although the domain containing all six phosphorylation sites is expected to be 90% disordered), and, in agreement with experimental results [34], Sic1 is predicted to be 80% disordered with a LDD of 93 residues. This level of disorder is unusual; the probability of randomly selecting four proteins of which three have a LDD of greater than 70 residues is approximately 0.01 for all eukaryotic proteins, rising to 0.05 when sampling is restricted to signaling proteins [16].

The interaction between Sic1 and SCF^{Cdc4} is perhaps the best studied. Sic1 is known to be disordered and polyvalent [34] and to bind to just a single phosphoepitope binding site on Cdc4 [30]. Each phosphorylated site on Sic1 has a low affinity for Cdc4, and binding only occurs when six or more sites are phosphorylated, irrespective of the order of phosphorylation [34]. The K_D between a single Sic1 site and Cdc4 is approximately 10^{-5} M in vivo [34] and thus a concentration of Cdc4 of 100 nM results in a $K_{eff} \approx 100$. Although a direct comparison with our model is not possible, we predict that such a large K_{eff} would give the highly cooperative binding observed (Figure 3, ignoring the zero phosphorylation point because binding occurs only when Sic1 is phosphorylated [34]). We predict a reduction in Sic1 disorder when it is phosphorylated. Structure is not apparent in (unpublished) NMR data of phosphorylated Sic1, indicating that phosphorylations do not perhaps induce a folded region but may only locally restrict protein flexibility. For example, a phosphorylated serine in the kinase inducible transactivation domain of CREB creates a small increase in helical content of the domain but does not lead to persistent secondary structure [37].

For general entropic, phosphorylation-driven binding, we predict that binding should become more cooperative as K_{eff} increases (Figure 3), either through decreasing the binding energy between *A* and *B* (increasing K_D by, for example, mutating the binding site on *B*) or through decreasing the concentration of B (Equation 3). The dependence of the cooperativity on the concentration of *B* is perhaps most intriguing and could be a biologically exploited control mechanism. In our simple model, a 10^4-fold increase in concentration leads to a 4-fold decrease in Hill coefficient (Figure 3). We also predict that the larger the disordered region of the interacting protein,

the more phosphorylations will be needed for complete binding and the more cooperative that binding will be (Figure 4). Similarly, deleting a disordered section of the protein should favor binding, providing the binding site itself is not also removed. Although we have emphasized the phosphorylation-induced local ordering necessary in our model, it is also possible that phosphorylation could instead disrupt local order. Cooperative binding would then occur as protein *A* is dephosphorylated rather than phosphorylated. Both phosphoserines [22] and phosphothreonines [38] can destabilize α helices, and serine phosphorylation induces a local change in phospholamban that propagates and ultimately causes a more global order-to-disorder transition [39]. An example of phosphorylation-inhibited binding is NFAT1, a human transcription factor. It is phosphorylated on at least 21 serines and requires dephosphorylation of 13 to enable nuclear import, i.e., binding to the nuclear transport apparatus [40].

Here, we have shown that if phosphorylations induce local order in a disordered protein, there is a nonlinear change in the protein's conformational entropy. As a function of the number of its phosphorylated sites, such a change can cause highly cooperative binding of the protein to a single binding site on an interacting protein. Multiple phosphorylations have been argued to generate cooperative protein responses if the phosphorylating kinase acts distributively (unbinds after each phosphorylation) [41] and sequentially (phosphorylating residues in a fixed order) [42]. The Hill coefficient describing this response is predicted to be less than the number of phosphorylated sites [42]. Our scheme generates greater cooperativity and does not rely on sequential phosphorylations or distributive kinase action: It depends only on changes in the intrinsic disorder of the phosphorylated substrate.

The cooperativity of entropic, phosphorylation-induced binding is so great that binding can become highly specific to the phosphorylated state of the protein; for example, if protein A is phosphorylated six times or less on any of its phosphorylation sites, only up to 10% of protein is bound to target, whereas if it is phosphorylated seven times or more, over 80% is bound (Figure 3 when $K_{eff} = 10^{-5}$). For those multiply phosphorylated proteins that require priming via a few phosphorylated residues before other residues can be phosphorylated, specificity could be maintained via kinetic proof-reading, where the energy of the priming phosphorylations is used to effectively enhance binding affinities [43]. Given its highly cooperative, and hence highly specific, nature and how cooperativity can be controlled through changes in concentration, it is tempting to think that entropic, phosphorylation-driven binding is a common cellular event.

Experimental Procedures

To calculate S^{conf} for a protein of a specified length with phosphorylation sites at specified locations, we wrote C^{++} code that exhaustively runs through all 3D, random walks of the appropriate length on a cubic lattice with six nearest neighbors. Each walk is checked for self-avoidance (two protein units cannot physically occupy the same space) and whether its conformation has one or more kinks at the appropriate phosphorylation sites, i.e., if the conformation goes through an appropriate right angle there—see Figure 2 for a 2D example. It is important that we only analyze self-avoiding random walks because then the size of the reduction in entropy depends on both the length of the protein and the number of phosphorylations. For random walks without self-avoidance and with *m* phosphorylations, the number of walks reduces by a factor of 6^m on a cubic lattice, independent of the length of the protein. The total number of self-avoiding conformations, the number with one kink at a phosphorylation site, the number with two kinks at phosphorylation sites, etc., is stored, and the conformational entropy calculated from Equation 4. We then use Equation 2 to calculate the binding curves for a given K_{eff} in Figures 3 and 4. Intrinsic protein disorder was predicted with PONDR VL-XT (Molecular Kinetics, Indianopolis, IN 46268 [35, 36, 44].

Supplemental Data

Supplemental Data include mathematical derivations and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/21/2150/DC1/.

Acknowledgments

We thank Derek Bowie and Kalle Gehring for useful comments. P.L. acknowledges support from the Fonds der Chemischen Industrie. P.S.S. is supported by the Natural Sciences and Engineering Research Council (Canada) and by a Tier II Canada Research Chair.

Received: June 13, 2006
Revised: August 14, 2006
Accepted: September 4, 2006
Published: November 6, 2006

References

1. Mammen, M., Choi, S.K., and Whitesides, G.M. (1998). Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors. Angew. Chem. *37*, 2754–2794.
2. Dyson, H.J., and Wright, P.E. (2005). Intrinsically unstructured proteins and their functions. Nat. Rev. Mol. Cell Biol. *6*, 197–208.
3. Landau, L.D., and Lifschitz, E.M. (1984). Statistical Physics (London: Butterworth-Heinemann).
4. Stryer, L. (1995). Biochemistry (New York: Freeman).
5. Weinberg, R.L., Veprintsev, D.B., and Fersht, A.R. (2004). Cooperative binding of tetrameric p53 to DNA. J. Mol. Biol. *341*, 1145–1159.
6. Monod, J., Wyman, J., and Changeux, J.P. (1965). On the nature of allosteric transitions: A plausible model. J. Mol. Biol. *12*, 88–118.
7. Koshland, D.E., Nemethy, G., and Filmer, D. (1966). Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochemistry *5*, 365–385.
8. Goldbeter, A., and Koshland, D.E. (1981). An amplified sensitivity arising from covalent modification in biological systems. Proc. Natl. Acad. Sci. USA *78*, 6840–6844.
9. Huang, C.Y., and Ferrell, J.E. (1996). Ultrasensitivity in the mitogen-activated protein kinase cascade. Proc. Natl. Acad. Sci. USA *93*, 10078–10083.
10. Ackers, G.K., Johnson, A.D., and Shea, M.A. (1982). Quantitative model for gene regulation by lambda phage repressor. Proc. Natl. Acad. Sci. USA *79*, 1129–1133.
11. Ferrell, J.E. (1998). How regulated protein translocation can produce switch-like responses. Trends Biochem. Sci. *23*, 461–465.
12. Ferrell, J.E. (1996). Tripping the switch fantastic: How a protein kinase cascade can convert graded inputs into switch-like outputs. Trends Biochem. Sci. *21*, 460–466.
13. Buchler, N.E., Gerland, U., and Hwa, T. (2005). Nonlinear protein degradation and the function of genetic circuits. Proc. Natl. Acad. Sci. USA *102*, 9559–9564.
14. Xiong, W., and Ferrell, J.E. (2003). A positive-feedback-based bistable 'memory module' that governs a cell fate decision. Nature *426*, 460–465.
15. Miller, P., Zhabotinsky, A.M., Lisman, J.E., and Wang, X.J. (2005). The stability of a stochastic CaMKII switch: Dependence

on the number of enzyme molecules and protein turnover. PLoS Biol. *3*, e107.
16. Iakoucheva, L.M., Brown, C.J., Lawson, J.D., Obradovic, Z., and Dunker, A.K. (2002). Intrinsic disorder in cell-signaling and cancer-associated proteins. J. Mol. Biol. *323*, 573–584.
17. Iakoucheva, L.M., Radivojac, P., Brown, C.J., O'Connor, T.R., Sikes, J.G., Obradovic, Z., and Dunker, A.K. (2004). The importance of intrinsic disorder for protein phosphorylation. Nucleic Acids Res. *32*, 1037–1049.
18. Cohen, P. (2000). The regulation of protein function by multisite phosphorylation - a 25 year update. Trends Biochem. Sci. *25*, 596–601.
19. Tholey, A., Lindemann, A., Kinzel, V., and Reed, J. (1999). Direct effects of phosphorylation on the preferred backbone conformation of peptides: A nuclear magnetic resonance study. Biophys. J. *76*, 76–87.
20. Shen, T., Wong, C.F., and McCammon, J.A. (2001). Atomistic Brownian dynamics simulation of peptide recognition. J. Am. Chem. Soc. *123*, 9107–9111.
21. Stultz, C.M., Levin, A.D., and Edelman, E.R. (2002). Phosphorylation-induced conformational changes in a mitogen-activated protein kinase substrate. Implications for tyrosine hydroxylase activation. J. Biol. Chem. *77*, 47653–47661.
22. Andrew, C.D., Warwicker, J., Jones, G.R., and Doig, A.J. (2002). Effect of phosphorylation on alpha-helix stability as a function of position. Biochemistry *41*, 1897–1905.
23. Bielska, A.A., and Zondlo, N.J. (2006). Hyperphosphorylation of tau induces local polyproline II helix. Biochemistry *45*, 5527–5537.
24. Suenaga, A., Kiyatkin, A.B., Hatakeyama, M., Futatsugi, N., Okimoto, N., Hirano, Y., Narumi, T., Kawai, A., Susukita, R., Koishi, T., et al. (2004). Tyr-317 phosphorylation increases Shc structural rigidity and reduces coupling of domain motions remote from the phosphorylation site as revealed by molecular dynamics simulations. J. Biol. Chem. *279*, 4657–4662.
25. Young, M.A., Gononi, S., Superti-Furga, G., Roux, B., and Kuriyan, J. (2001). Dynamic coupling between the SH2 and SH3 domains of c-Src and Hck underlies their inactivation by C-terminal tyrosine phosphorylation. Cell *105*, 115–126.
26. Wulf, G., Finn, G., Suizu, F., and Lu, K.P. (2005). Phosphorylation-specific prolyl isomerization: Is there an underlying theme? Nat. Cell Biol. *7*, 435–441.
27. Yaffe, M.B., Schutkowski, M., Shen, M., Zhou, X.Z., Stukenberg, P.T., Rahfeld, J.U., Xu, J., Kuang, J., Kirschner, M.W., Fischer, G., et al. (1997). Sequence-specific and phosphorylation-dependent proline isomerization: A potential mitotic regulatory mechanism. Science *278*, 1957–1960.
28. Stukenberg, P.T., and Kirschner, M.W. (2001). Pin1 acts catalytically to promote a conformational change in Cdc25. Mol. Cell *7*, 1071–1083.
29. Busino, L., Chiesa, M., Draetta, G.F., and Donzelli, M. (2004). Cdc25A phosphatase: Combinatorial phosphorylation, ubiquitylation and proteolysis. Oncogene *23*, 2050–2056.
30. Orlicky, S., Tang, X., Willems, A., Tyers, M., and Sicheri, F. (2003). Structural basis for phosphodependent substrate selection and orientation by the SCFCdc4 ubiquitin ligase. Cell *112*, 243–256.
31. Chi, Y., Huddleston, M.J., Zhang, X., Young, R.A., Annan, R.S., Carr, S.A., and Deshaies, R.J. (2001). Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. Genes Dev. *15*, 1078–1092.
32. Hsu, J.M., Lee, Y.C., Yu, C.T., and Huang, C.Y. (2004). Fbx7 functions in the SCF complex regulating Cdk1-cyclin B-phosphorylated hepatoma up-regulated protein (HURP) proteolysis by a proline-rich region. J. Biol. Chem. *279*, 32592–32602.
33. Schwartz, M.F., Duong, J.K., Sun, Z., Morrow, J.S., Pradhan, D., and Stern, D.F. (2002). Rad9 phosphorylation sites couple Rad53 to the Saccharomyces cerevisiae DNA damage checkpoint. Mol. Cell *9*, 1055–1065.
34. Nash, P., Tang, X., Orlicky, S., Chen, Q., Gertler, F.B., Mendenhall, M.D., Sicheri, F., Pawson, T., and Tyers, M. (2001). Multisite phosphorylation of a CDK inhibitor sets a threshold for the onset of DNA replication. Nature *414*, 514–521.
35. Li, X., Romero, P., Rani, M., Dunker, A.K., and Obradovic, Z. (1999). Predicting protein disorder for N-, C-, and internal regions. Genome Inform. Ser. Workshop Genome Inform. *10*, 30–40.
36. Romero, P., Obradovic, Z., Li, X., Garner, E.C., Brown, C.J., and Dunker, A.K. (2001). Sequence complexity of disordered protein. Proteins *42*, 38–48.
37. Radhakrishnan, I., Perez-Alvarado, G.C., Dyson, H.J., and Wright, P.E. (1998). Conformational preferences in the Ser133-phosphorylated and non-phosphorylated forms of the kinase inducible transactivation domain of CREB. FEBS Lett. *430*, 317–322.
38. Szilak, L., Moitra, J., Krylov, D., and Vinson, C. (1997). Phosphorylation destabilizes alpha-helices. Nat. Struct. Biol. *4*, 112–114.
39. Metcalfe, E.E., Traaseth, N.J., and Veglia, G. (2005). Serine 16 phosphorylation induces an order-to-disorder transition in monomeric phospholamban. Biochemistry *44*, 4386–4396.
40. Okamura, H., Aramburu, J., Garcia-Rodriguez, C., Viola, J.P., Raghavan, A., Tahiliani, M., Zhang, X., Qin, J., Hogan, P.G., and Rao, A. (2000). Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. Mol. Cell *6*, 539–550.
41. Ferrell, J.E., and Bhatt, R.R. (1997). Mechanistic studies of the dual phosphorylation of mitogen-activated protein kinase. J. Biol. Chem. *272*, 19008–19016.
42. Salazar, C., and Hofer, T. (2003). Allosteric regulation of the transcription factor NFAT1 by multiple phosphorylation sites: A mathematical analysis. J. Mol. Biol. *327*, 31–45.
43. Swain, P.S., and Siggia, E.D. (2002). The role of proofreading in signal transduction specificity. Biophys. J. *82*, 2928–2933.
44. Romero, P., Obradovic, Z., and Dunker, A.K. (1997). Sequence data analysis for long disordered regions prediction in the calcineurin family. Genome Inform. Ser. Workshop Genome Inform. *8*, 110–124.