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Productive induced metastability in allosteric modulation of kinase function

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Allosteric modulators of kinase function are of considerable pharmacological interest as blockers or agonists of key cell-signaling pathways. They are gaining attention due to their purported higher selectivity and efficacy relative to ATP-competitive ligands. Upon binding to the target protein, allosteric inhibitors promote a conformational change that purposely facilitates or hampers ATP binding. However, allosteric binding remains a matter of contention because the binding site does not fit with a natural ligand (i.e. ATP or phosphorylation substrate) of the protein. In this study, we show that allosteric binding occurs by means of a local structural motif that promotes association with the ligand. We specifically show that allosteric modulators promote a local metastable state that is stabilized upon association. The induced conformational change generates a local enrichment of the protein in the so-called dehydrons, which are solvent-exposed backbone hydrogen bonds. These structural deficiencies that are inherently sticky are not present in the apo form and constitute a local metastable state that promotes association with the ligand. This productive induced metastability (PIM) is likely to translate into a general molecular design concept.

Introduction

In this work, we show that allosteric modulation of kinase function arises from the creation of a structural vulnerability that promotes and is corrected by association of the protein with the allosteric ligand. The vulnerable state is metastable because it is enriched in packing defects, the so-called dehydrons [1–3], which are solvent-exposed backbone hydrogen bonds. These induced intramolecular hydrogen bonds promote their own dehydration and therefore drag the binding partner into their proximity [1–8]. Thus, this application illustrates that intramolecular hydrogen bonds are not only determinants of protein structure, but also promoters of protein association and functional modualtion. At variance with previous work on dehydron physics [1–8], the allosteric dehydrons described here are dynamically induced through allosteric conformational change and not are pre-existent.

Kinase inhibitors and agonists have received considerable attention from the molecular pharmaceutics community because of the therapeutic value of blocking or enhancing specific cell-signaling pathways in a disease-related context [9–27]. This therapeutic opportunity opened up with the realization that key signal transducers, the protein kinases, possess binding pockets for their natural ligand (ATP) that may be targeted by small molecules. However, ATP-binding sites are populated by structural moieties that are highly conserved across the human kinome, posing big challenges to the molecular designer that aims at a target-selective therapeutic agent. Furthermore, an ATP-competitive inhibitor is only pharmacologically rele-

Abbreviations
AKT1, RAC-alpha serine/threonine-protein kinase; CDK2, Cyclin-dependent kinase 2; KIT, c-Kit proto-oncogene protein tyrosine kinase; PDK1, 3-phosphoinositide dependent protein kinase-1; PIM, productive induced metastability.

vant if its K_d is in the nanomolar range because it needs to displace the natural ligand to become operative. These shortcomings have motivated researchers to pursue allosteric inhibitors or agonists capable of exacerbating or hampering ATP binding through association at a distal site on the target protein [9–27]. Distal binding promotes a conformational change that alters the affinity for ATP. The binding of such allosteric ligands (inhibitors or agonists) remains a matter of controversy because the site of the association is difficult to predict and rationalize upon direct examination of the ligand–protein interface [28–30]. In this, work we examine such allosteric interfaces for a number of kinases including c-Kit proto-oncogene protein tyrosine kinase (KIT), interleukin-2-inducible T-cell kinase (ITK), vascular endothelial growth factor receptor (VEGFR), 3-phosphoinositide dependent protein kinase-1 (PDK1), RAC-alpha serine/threonine-protein kinase (AKT1) and cyclin-dependent kinase 2 (CDK2). In all cases, we find a structurally induced metastable state that gains stabilization through ligand binding. We name this mode association productive induced metastability (PIM). In concrete terms, the metastability is brought about by local enrichment of the protein structure in packing defects that become 'corrected' upon association with the ligand. These defects are not present in the apo form of the protein. In plain terms, the protein becomes 'needy' for the ligand through local structural degradation that promotes formation of the complex.

To prevail in water environments, soluble proteins protect their backbone hydrogen bonds (hydrogen bonds) from the disruptive effect of water attack by clustering nonpolar residues around them. This exclusion of the surrounding water, or wrapping effect [1–8], also enhances the electrostatic contribution by modulating the local dielectric (descreening the partial charges) and thus stabilizes the hydrogen bond. In turn, and as demonstrated previously, underwrapped interactions represent packing defects, the so-called dehydrons, where the surrounding water has not been properly excluded; being thus adhesive, dehydrons promote protein associations because their inherent stability increases upon the approach of additional nonpolar residues [1–8]. The packing defects induced by the allosteric ligand, of interest here, are the dehydrons [1–8]. Dehydrons, as poorly wrapped solvent-exposed backbone hydrogen bonds, can only be properly solvated by creating interfacial tension. This means that water molecules hydrogen bonding to the paired backbone amide or carbonyl can only do so by relinquishing some level of hydrogen bonding coordination with vicinal groups or water molecules. These structural singularities constitute vulnerabilities in the structure because backbone hydration is a disruptor of the folded state of the protein. By displacing the tension-creating interfacial water upon binding, the ligand stabilizes the protein when incorporated into a protein–ligand complex. In other words, dehydrons are metastable states flanked by two more stable local conformations, respectively the locally unfolded state and the well-wrapped state with its hydrogen bond completely sequestered from water given the shielding effect of the surrounding nonpolar groups. The unfolded state is more stable than the dehydron state because of the unhampered hydration of the backbone and its higher entropic content, which results from the removal of a constraint. However, the well-wrapped state becomes more stable than the dehydron because burial of the partial charges of the amide and carbonyl removes the local screening and destabilizes the nonbonded state with its partial charges deprived from hydration (the experimentally determined ΔG (Gibbs free energy) of wrapping one dehydron is 3.9 $kJ \cdot mol^{-1}$ [1,2]). These dehydrons are a common occurrence in protein structures and result from packing defects that

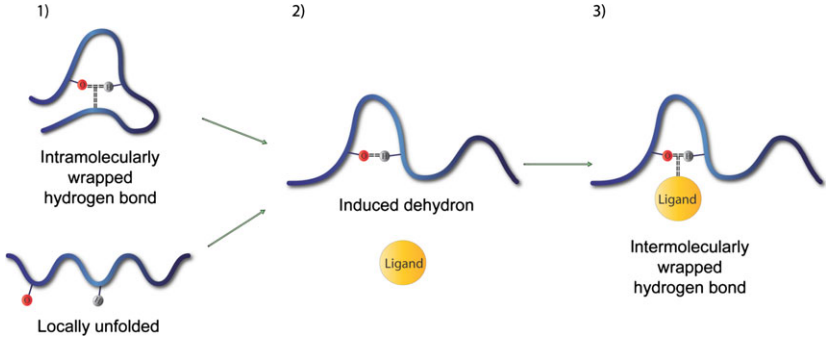


Fig. 1. The two types of productive induced metastability (PIM) events. Starting from a well-wrapped or locally unfolded structure, a dehydron is created which is in turn intermolecularly wrapped by the ligand.

fail to completely exclude water from the backbone. As is well established, such dehydrons can be readily inferred from the structural coordinates of PDB-reported structures and usually signal-binding hotspots in the interfaces of protein associations [1,7]. These associations materialize by intermolecular wrapping of preformed dehydrons in one of the binding partners.

In this work, we emphasize a different binding mode wherein the dehydron is not preformed or pre-existent in the target protein, but is induced as the ligand binds to it. The conformational ensemble in the apo form of the protein is unlikely to have the dehydron-rich conformation as a statistically significant microstate. Here, we identify two microstates that have a higher predominance and hence are represented in the crystal structure of the apo form of the protein: the locally unfolded state in which backbone amides and carbonyls are not paired, and the well-wrapped state in which the backbone hydrogen bond is sequestered from water. Thus, the structural effect of the allosteric ligand may be characterized as either an inducer of a dehydron from a partially unfolded state or a structural degrader wherein a well-wrapped hydrogen bond becomes a dehydron. In both cases, the resulting dehydron-enriched locally metastable state prompts association with the ligand as a means to achieve further stabilization through intermolecular wrapping Fig. 1. We have thus provided a structural description of the PIM, the mode of association of an allosteric modulator of kinase activity.

Results and discussion

Here, we investigate the mechanism of allosteric modulation in different kinases in order to highlight the main role of PIM events. To identify PIM events, we compare the wrapping patterns of the apo form of the kinase with that of the kinase–ligand complex. In the complex, we discriminate between intramolecular wrapping (ρ-values without ligand contribution) and the total ρ-values when the ligand contribution is included. The conformation of the kinase allosterically stabilized by the ligand is expected to be different from the apo form and thus the intramolecular wrapping is different in both conformations.

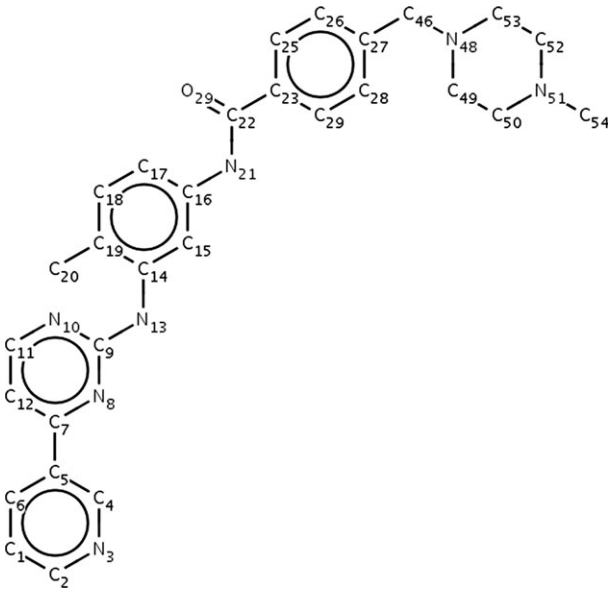
The first example (Table 1, Scheme 1 and Fig. 2) corresponds to an interesting case of allosteric modulation involving kinases that can exist in both a DFG-in and DFG-out conformation. The DFG-in conformation is the active one because it binds ATP, whereas the DFG-out conformation sterically blocks the access of ATP to the active site within the activation loop. Several drugs (like imatinib [14–17,20] and sunitinib [18,20–22]) have been found to bind to the DFG-out conformation at allosteric sites, thus inhibiting kinase activity. In Table 1 and Fig. 2, we show the case of KIT kinase in complex with the drug imatinib (PDB codes 1PKG for the kinase in the DFG-in conformation [14], which does not bind imatinib, and 1T46 for the kinase in the DFG-out conformation in complex with imatinib [15]).

Table 1. Study of the complex between KIT and the drug imatinib.

Hydrogen bond	Apo enzyme ρ	Inhibited enzyme ρ without ligand	Inhibited enzyme ρ with ligand	Wrappers in ligand
Leu595–Val603	20	**19**	22	C1 C6 C20
Gly596–Val603	**18**	21	22	C20
Val603–Gly596	**18**	21	22	C20
Val604–Val622	30	26	29	C19 C18 C20
Ala621–Thr670	32	27	31	C5 C19 C18 C20
Val622–Val604	30	26	29	C19 C18 C20
Lys623–Val668	24	25	29	C19 C18 C17 C20
Glu640–Ala636	**19**	**17**	**19**	C28 C29
Val643–Ser639	24	22	23	C28
Leu644–Glu640	23	**19**	22	C27 C28 C29
Leu647–Val643	27	27	28	C28
Gly648–Leu644	21	**16**	**19**	C27 C28 C29
Val654–Ile808	26	23	25	C15 C25
Leu657–Ile669	28	29	32	C19 C18 C20
Gly658–Ile669	*	31	34	C19 C18 C20
Val668–Lys623	24	25	29	C19 C18 C17 C20
Ile669–Gly658	*	31	34	C19 C18 C20
Thr670–Ala621	32	27	31	C5 C19 C18 C20
Gly676–Cys673	*	**16**	**19**	C1 C6 C5
Phe681–Asp677	21	23	25	C1 C6
Leu800–Gly676	*	20	23	C1 C6 C5
Ile808–Asn652	*	23	24	C25
Cys809–Asn797	23	**19**	25	C15 C23 C25 C26 C27 C29
	PDB code 1PKG	PDB code 1T46		

Bold values indicate HBs with ρ number below the dehydron threshold (ρ < 20); Shaded rows indicate the existence of a PIM event. *Indicate that the Hydrogen bond is not formed in the corresponding PDB structure.

As in all the tables, Table 1 includes all the intramolecular hydrogen bonds of the kinase whose wrapping values change upon association with the allosteric modulator. For each hydrogen bond, Table 1 shows the residues paired by the hydrogen bond, and its ρ-values in the apo form and in the complex. An asterisk indicates that the hydrogen bond is not formed in the corresponding PDB structure (in some cases, it also indicates a disordered region in which the corresponding residues are not resolved in the PDB structure). Dehydrons are indicated by giving the ρ-value of the



Scheme 1. STI (IMATINIB) molecular structure with PDB atom labels.

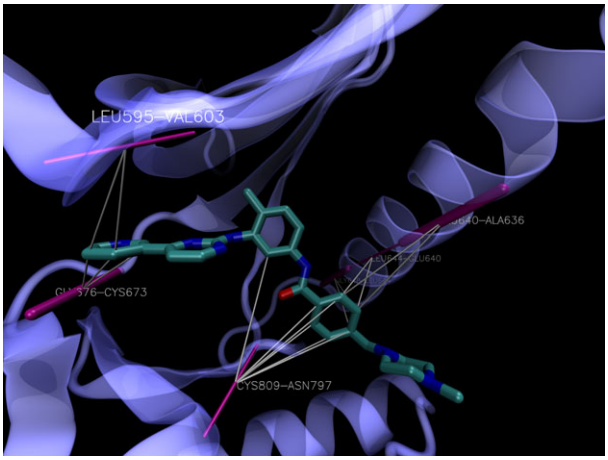


Fig. 2. Three-dimensional structure of the complex between KIT and imatinib, depicting PIMs and direct-wrapping events.

hydrogen bonds in the corresponding structure in bold (those with $\rho < 20$). A PIM event is indicated by shading in the corresponding row of the table. Numbering for heavy atoms of the ligand are given as indicated in the ligand structure representation in Scheme 1.

For a PIM event to occur, three conditions should be met:

- The ρ-value of a hydrogen bond within the complex is $\rho < 20$ when excluding the ligand contribution. In other words, the kinase conformation within the complex presents a dehydron as the complex is dissociated *in silico*.
- Either (a) the hydrogen bond in the apo form presents a ρ-value ≥ 20 (well-wrapped hydrogen bond) or (b) the hydrogen bond is not formed in the apo form. In both cases the dehydron is not pre-existent in the apo form but is induced upon binding. Case (a) corresponds to the degradation of a well-wrapped hydrogen bond, whereas in case (b) a hydrogen bond is induced from a locally unfolded state.
- The wrapping contribution of the allosteric ligand increases the ρ-value of the degraded or induced hydrogen bond upon association. A stronger condition implies that the binding should increase ρ above the dehydron threshold ($\rho \geq 20$).

In turn, in Fig. 2 we present the 3D structure of the complex of the kinase with its allosteric modulator. As in all forthcoming 3D structure representations in this article, the thin white lines drawn from the ligand to the kinase (in the complex) indicate wrapping interactions provided by the group of the ligand where the line originates. The bars in the kinase (in the complex) structure indicate dehydrons pre-existent in the apo form (light gray, green in the online version) or PIMs (dark gray, magenta in the online version). In both cases, thin bars indicate that the hydrogen bond reaches a ρ-value above the dehydron threshold when wrapped by the ligand, whereas thick bars indicate that the hydrogen bond (from a pre-existent dehydron or a PIM) remains as a dehydron when we also add the wrapping provided by the ligand. Thus, a thick bar represents the existence of regions of the ligand that could be re-engineered (by inclusion of additional wrapper groups) to completely remove the dehydron or local metastability. In all cases, bars are represented connecting α carbons of the residues that form the hydrogen bonds.

Table 1 makes evident the main role of PIM events in the allosteric binding of imatinib. Table 1 and Fig. 2 show that this allosteric drug binds to the DFG-out conformation of KIT kinase by means of six PIM events. One such example is that of the hydrogen bond between Leu595 and Val603, which in the apo form is well wrapped, $\rho = 20$, although its intramolecular ρ-value decreases to $\rho = 19$ (dehydron) in the conformation of the kinase that binds the drug. In turn, the intermolecular wrapping provided by imatinib (by means of carbons C1, C6 and C20) increases the total ρ-value of such hydrogen bond to $\rho = 22$ (therefore removing the dehydron). An example of the other type of PIM event is provided by the hydrogen bond between Gly676 and Cys673. This hydrogen bond is not formed

in the apo form, but is induced in the allosteric complex under the following conditions. The intramolecular wrapping of this hydrogen bond (ρ = 16) falls below the dehydron threshold in the allosteric conformation of the kinase, but amounts to ρ = 19 if we consider the intermolecular wrapping provided by the ligand within the complex (it is brought to the dehydron threshold value). It is interesting to note that the hydrogen bonds of three of such PIMs amount to a wrapping value of ρ = 19 (exactly at the dehydron threshold) when we consider the intermolecular wrapping provided by imatinib. Thus, we expect that addition of wrapper groups in the surroundings of the appropriate carbon atoms (C27, C28, C29, C1, C5 and C6, as indicated in Table 1) might improve the binding. Such a PIM-guided re-engineering might aid future design efforts. In turn, imatinib only wraps a couple of dehydrons pre-existent in the apo form. Figure 2 illustrates the wrapping interactions described for such complex, both involving PIMs and wrapping of pre-existing dehydrons.

In turn, the case of the complex between kinase p38 and imatinib is also instructive. It is known that imatinib is not a good inhibitor of this kinase [16,17]. Consistently, if we perform this type of analysis, we can only find one PIM event, as shown in Table 2.

The case of the binding of the drug sunitinib to the kinase VEGR2 also conforms to the context of the allosteric modulation by stabilization of kinase DFG-out conformation. Table 3, Scheme 2 and Fig. 3 illustrate this situation (PDB codes: 1VR2 [18] for the kinase and 4AGD [19] for the complex). In this case, the drug binds the DFG-out conformation of the kinase by means of four direct-wrapping events and two PIMs. The PIM wrapped by the carbons C5 and C7 of the drug might be further improved by including an additional wrapper group at such positions.

In the inhibition of KIT by the binding of sunitinib to its DFG-out conformation (PDB codes: 3GOE [20] for the kinase and 1PKG [14] for the complex), we

Table 2. P38 kinase in complex with imatinib.

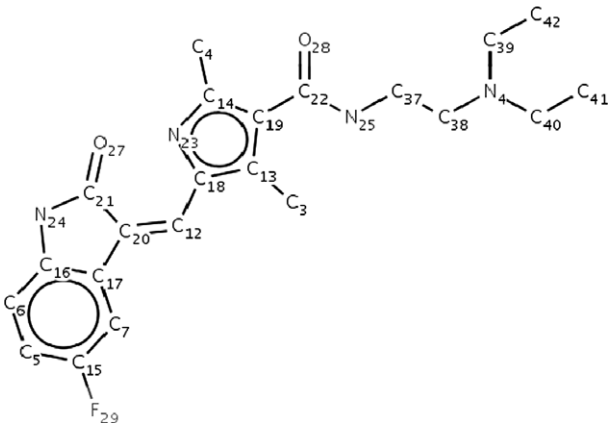
Hydrogen bond	Apo enzyme ρ	Inhibited enzyme ρ without ligand	Inhibited enzyme ρ with ligand	Wrappers in ligand
Cys39–Val52	24	24	26	C19 C20
Ala51–Thr106	24	24	27	C5 C19 C20
Val52–Cys39	24	24	26	C19 C20
Lys53–Leu104	27	29	33	C19 C18 C17 C20
Leu104–Lys53	27	29	33	C19 C18 C17 C20
Val105–Asp88	28	27	30	C19 C18 C20
Thr106–Ala51	24	24	27	C5 C19 C20
Leu108–Arg49	*	**18**	20	C1 C5
Leu167–Asn155	22	22	28	C15 C23 C25 C27 C28 C29
	PDB code 3PY3	PDB code 3HEC		

Bold values indicate HBs with ρ number below the dehydron threshold (ρ < 20); Shaded rows indicate the existence of a PIM event. *Indicate that the Hydrogen bond is not formed in the corresponding PDB structure.

Table 3. Study of the complex between VEGR2 and sunitinib.

Hydrogen bond	Apo enzyme ρ	Inhibited enzyme ρ without ligand	Inhibited enzyme ρ with ligand	Wrappers in ligand
Leu840–Val848	**17**	**18**	22	C13 C3 C12 C19
Gly841–Val848	**15**	**19**	21	C13 C3
Val848–Gly841	**15**	**19**	21	C13 C3
Ala866–Val916	31	30	31	C6
Val899–Ile1044	29	23	24	C6
Val916–Ala866	31	30	31	C6
Glu917–Asn900	21	20	21	C6
Phe921–Leu1036	22	24	25	C4
Gly922–Cys919	*	**16**	22	C4 C13 C20 C3 C12 C19
Leu1036–Gly922	**16**	**16**	22	C4 C13 C20 C3 C12 C19
Cys1045–Asn1033	24	24	26	C5 C6
Ala1050–Phe1047	*	**14**	**16**	C5 C7
	PDB code 1VR2	PDB code 4AGD		

Bold values indicate HBs with ρ number below the dehydron threshold (ρ < 20); Shaded rows indicate the existence of a PIM event. *Indicate that the Hydrogen bond is not formed in the corresponding PDB structure.



Scheme 2. SUNITINIB molecule labeled as in the PDB.

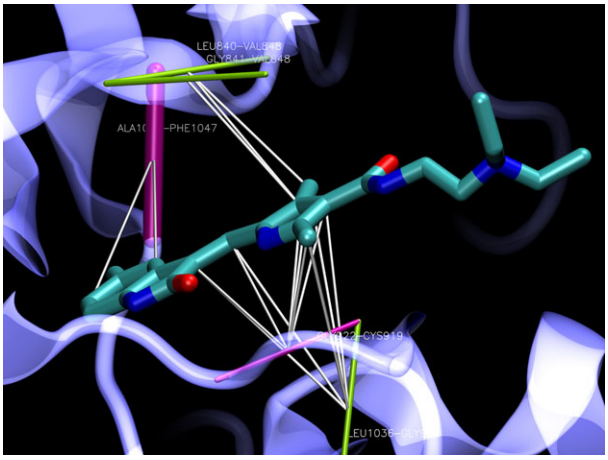


Fig. 3. Three-dimensional representation of the complex VEGR2–sunitinib, indicating PIMs and direct-wrapping events.

Table 4. KIT kinase in complex with sunitinib.

Hydrogen bond	Apo enzyme ρ	Inhibited enzyme ρ without ligand	Inhibited enzyme ρ with ligand	Wrappers in ligand
Leu595–Val603	20	21	27	C7 C13 C20 C3 C12 C19
Gly596–Val603	**18**	22	24	C7 C3
Val603–Gly596	**18**	22	24	C7 C3
Ala621–Thr670	32	27	28	C6
Thr670–Ala621	32	27	28	C6
Tyr675–Leu800	25	22	23	C4
Gly676–Cys673	*	**16**	22	C4 C13 C20 C3 C12 C19
Leu800–Gly676	*	**19**	24	C4 C13 C3 C12 C19
Cys809–Asn797	23	23	24	C5
Ala814–Phe811	*	**17**	**19**	C5 C7
	PDB code 1PKG	PDB code 3G0E		

Bold values indicate HBs with ρ number below the dehydron threshold (ρ < 20); Shaded rows indicate the existence of a PIM event. *Indicate that the Hydrogen bond is not formed in the corresponding PDB structure.

found two direct-wrapping events and three PIMs (Table 4). One of such PIM is suboptimal because further wrapping would be required to bring the hydrogen bond above the dehydron threshold.

A similar situation is shown in Table 5 for the binding of the inhibitor drug sunitinib to ITK (PDB codes: 1SNX [21] for the kinase and 3MIY [22] for the complex). One direct-wrapping event and three PIMs are found (two such PIMs are suboptimal and might

Table 5. ITK kinase in complex with sunitinib.

Hydrogen bond	Apo enzyme ρ	Inhibited enzyme ρ without ligand	Inhibited enzyme ρ with ligand	Wrappers in ligand
Ile369–Val377	**14**	**14**	**17**	C13 C3 C19
Gly370–Val377	*	**13**	**15**	C13 C3
Val377–Gly370	**12**	**13**	**15**	C13 C3
Ala389–Phe435	35	34	40	C5 C6 C7 C17 C20 C12
Phe435–Ala389	35	34	40	C5 C6 C7 C17 C20 C12
Glu436–Gln420	22	21	22	C6
Gly441–Met438	*	**17**	24	C4 C13 C20 C3 C12 C19 C42
Tyr446–Cys442	22	22	25	C13 C3 C12
Val490–Gly441	**17**	**16**	23	C4 C13 C20 C3 C12 C19 C42
Ser499–Asn487	*	**17**	**18**	C5
	PDB code 1SNX	PDB code 3MIY		

Bold values indicate HBs with ρ number below the dehydron threshold (ρ < 20); Shaded rows indicate the existence of a PIM event. *Indicate that the Hydrogen bond is not formed in the corresponding PDB structure.

present re-engineering opportunities). In addition, a couple of pre-existing dehydrons could be better wrapped by the drug to bring the ρ-values of the corresponding hydrogen bonds above the dehydron threshold.

Another example we considered (PDB entries: 1PW2 [23] for the kinase and 1FIN [24] for the complex) is the binding of the activator protein cyclin A to the kinase CDK2. Four PIM events were detected in such study, displayed in Table 6.

Another excellent example is provided by the allosteric modulation of kinase AKT1 by a peptidic activator (PDB codes 3O96 [12] and 4EKK [13]). As illustrated in Table 7 (Scheme 3), in this case we found eight PIMs and no direct-wrapping event.

The final set of examples studied corresponds to the allosteric binding to the PIF pocket of the kinase PDK1. This pocket is quite distal from the activation loop. We studied in Table 8 (Scheme 4) the activation of PDK1 by the ligand 2A2 (PDB codes: 1H1W [25] for the kinase and 3ORZ [26] for the complex). In this case, we found one PIM event and a direct-wrapping event, although another dehydron could be better wrapped by modification of the ligand. We also analyzed the case of PDK1 activated by the ligand Ps-171 (AZ7 in the PDB) (PDB 4A07 [26a] for the complex). Here, we found one PIM and one direct-wrapping

Table 6. CDK2 kinase in complex with protein cyclin A.

Hydrogen bond	Apo enzyme ρ	Activated enzyme ρ without ligand	Activated enzyme ρ with ligand	Wrappers in ligand
Glu40–Asp38	*	**12**	**18**	CB CG CD CB CG CD2
Glu42–Asp38	*	**11**	**16**	CB CG2 CG CD1 CD2
Gly43–Asp38	*	**13**	**19**	CG2 CB CG CD1 CD2 CG
Ile49–PRO45	20	**17**	22	CD CD1 CE1 CZ CD1
Arg50–Ser46	**15**	**16**	22	CB CG CD CG CD1 CE1
Ile52–Ala48	27	29	33	CB CG CD1 CE1
Ser53–Ile49	24	20	30	CE1 CB CG CD1 CE1 CZ CB CG CD1 CB
Leu54–Arg50	**18**	20	24	CG CD1 CE1 CB
Lys56–Ile52	*	21	25	CB CG CD1 CE1
Glu57–Leu54	**15**	**15**	**17**	CE1 CB
	PDB code 1PW2	PDB code 1FIN		

Bold values indicate HBs with ρ number below the dehydron threshold (ρ < 20); Shaded rows indicate the existence of a PIM event. *Indicate that the Hydrogen bond is not formed in the corresponding PDB structure.

Table 7. AKT1 kinase in complex with an activator peptide.

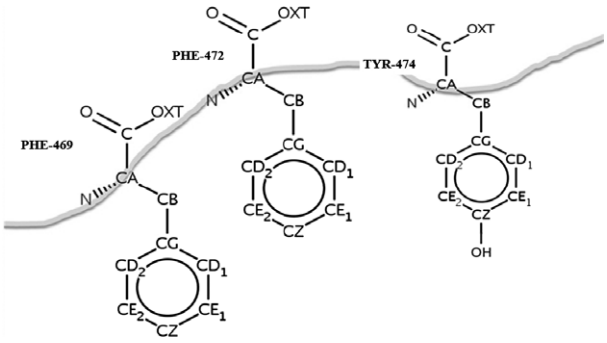
Hydrogen bond	Apo enzyme ρ	Activated enzyme ρ without ligand	Activated enzyme ρ with ligand	Wrappers in ligand
Leu181–Leu223	22	20	24	CE1-472 CZ-472 CE2-472 CD2-472
Lys183–Asp221	21	**18**	22	CB-466 CG-466 CE1-469 CZ469
Val187–Lys183	*	**17**	20	CG-466 CE1-469 CZ-469
Ala188–Glu184	*	**13**	**16**	CB-465 CG-465 CG-466
Leu196–Val192	*	**18**	23	CZ-469 CE2-469 CD2-469 CD1-472 CE1-472
Asn199–Thr195	*	24	27	CD1-472 CE1-472 CZ-472
Arg200–Leu196	*	**17**	**19**	CD1-472 CE1-472
Gln203–Asn199	*	**18**	26	CD1-472 CE1-472 CZ-472 CG-474 CD1-474 CE1-474 CE2-474 CD2-474
Lys214–Val226	*	23	26	CE2-474 CD2-474 CB-476
Tyr215–Val226	25	24	28	CB-474 CG-474 CE2-474 CD2-474
Phe217–Cys224	*	**19**	24	CG-472 CZ-472 CE2-472 CD2-472 CB-473
Leu223–Leu181	22	20	24	CE1-472 CZ-472 CE2-472 CD2-472
Cys224–Phe217	24	**19**	24	CG-472 CZ-472 CE2-472 CD2-472 CB-473
Val226–Tyr215	25	24	28	CB-474 CG-474 CE2-474 CD2-474
	PDB code 3O96	PDB code 4EKK		

Bold values indicate HBs with ρ number below the dehydron threshold (ρ < 20); Shaded rows indicate the existence of a PIM event. *Indicate that the Hydrogen bond is not formed in the corresponding PDB structure.

event, with two other possible direct-wrapping events if the ligand could provide additional wrapping to a pair of pre-existing dehydrons, as shown in Table 9 (Scheme 5). The fact that in our study, PDK1 showed fewer PIM events than the similar kinases AKT1 and AKT2 may be due to the following: the 3D structure of the protein crystallized by Biondi's group [27] has been suggested to present the PIF pocket region much more 'ordered' than it would actually be in solution. Supplementary information in Hindie *et al.* [27] indicates that there is experimental evidence (e.g. deuterium exchange) of a higher degree of exposure to solvent than that detected by X–ray crystallography. As reported, the PIF pocket could be linked to another portion of the protein in the crystal, thus protecting it from water. These considerations are consistent with the differences in the amount of PIM events we found for PDK1 as compared to AKT PIF pockets.

Before concluding, we briefly relate our findings to a general theory of allosteric function [31–37]. It was suggested long ago that the high efficiency of enzymes is a mechanical consequence of their ability to perform conformational changes or structural distortions, given the availability of alternative conformational substrates [31–37]. Such rich protein intramolecular mobility allows allosteric signaling to proceed by means of activated transitions between 'mobile-defect' conformers [31–37]. In addition, distortions of the local structure, also including changes in the hydrogen bond pattern, have been suggested to be the driving force for such fluctuations among different internal conformations [31–37]. Although our PIM description pertains to such general scenario it identifies a specific structural event, the PIM, responsible for allosteric modulation.

As a final point, it would be very interesting to determine whether there is a correlation between the number and/or quality of PIM events and binding

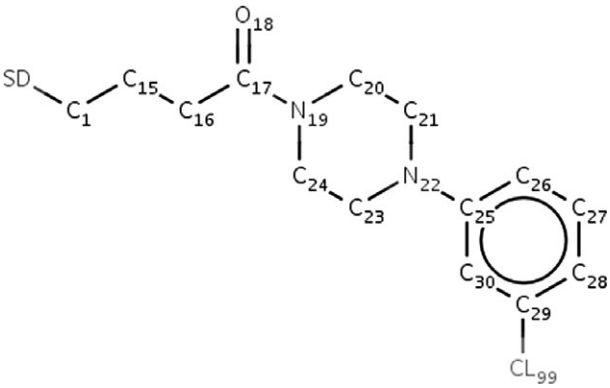


Scheme 3. Schematic diagram of the AKT1 activator peptide, with the key wrapping residues labeled as in the PDB.

Table 8. PDK1 kinase in complex with activator 2A2.

Hydrogen bond	Apo enzyme ρ	Activated enzyme ρ without ligand	Activated enzyme ρ with ligand	Wrappers in ligand
Lys115–Glu153	23	20	23	C27 C28 C30
Ile119–Lys115	21	**16**	**19**	C27 C28 C30
Asn122–Ile119	**16**	**15**	**16**	C28
Thr128–Val124	**19**	**19**	20	C30
	PDB code 1H1W	PDB code 3ORZ		

Bold values indicate HBs with ρ number below the dehydron threshold (ρ < 20); Shaded rows indicate the existence of a PIM event. *Indicate that the Hydrogen bond is not formed in the corresponding PDB structure.



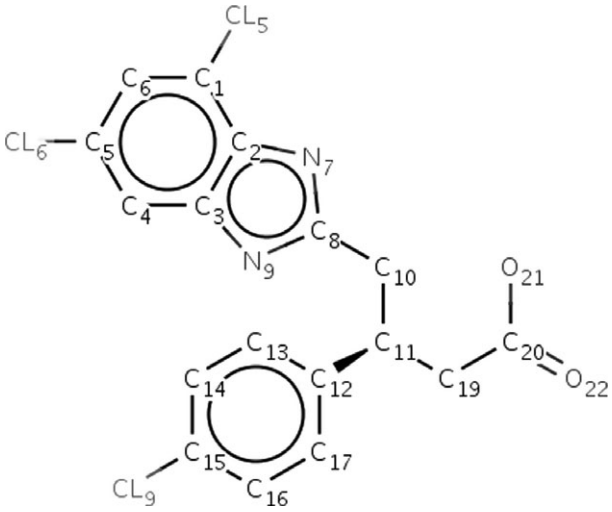
Scheme 4. PDK1 ligand structure labeled as in PDB.

affinity. The problem is that, even when there exist studies of the binding of different ligands to a given kinase, it is very difficult to perform a comparison because PIMs are not the only interactions responsible

Table 9. PDK1 in complex with allosteric activator PS-171.

Hydrogen bond	Apo enzyme ρ	Activated enzyme ρ without ligand	Activated enzyme ρ with ligand	Wrappers in ligand
Leu113–Leu155	27	25	28	C17 C16 C14
Lys115–Glu153	23	22	23	C6
Ile119–Lys115	21	**19**	20	C6
Thr128–Val124	**19**	20	23	C6 C14 C13
Arg131–Val127	23	22	23	C14
Asp132–Thr128	**14**	**13**	**15**	C14 C13
Ser135–Arg131	**16**	**16**	**17**	C14
Phe149–Tyr156	30	29	31	C17 C16
Leu155–Leu113	27	25	28	C17 C16 C14
Tyr156–Phe149	30	29	31	C17 C16
	PDB code 1H1W	PDB code 4A07		

Bold values indicate HBs with ρ number below the dehydron threshold (ρ < 20); Shaded rows indicate the existence of a PIM event. *Indicate that the Hydrogen bond is not formed in the corresponding PDB structure.



Scheme 5. Chemical structure of the ligand PS-171 (AZ7 in PDB), with its corresponding atom labels.

for the stability of the complex. Thus, it is not possible to isolate a single effect to view incrementally. Even the sizes of the different ligands may vary significantly from each other. However, such a comparison is indeed possible in the case of KIT kinase in complex with drugs re-engineered from imatinib by adding a methyl group to imatinib in different positions [38,39]. These represent ideal test cases because we can focus on the effect of a single change within a common scaffold.

The first case corresponds to the re-engineering of imatinib into WBZ_4 by the addition of a methyl group at carbon C2. This molecule was engineered [38] to be more active and less cardiotoxic. The latter goal was accomplished by the fact that this additional methyl avoids binding to Abl kinase by means of a steric effect, a target that is indeed inhibited by imatinib [38]. However, the increase in binding affinity to KIT kinase [38] can be explained by a PIM event. As already indicated, when we studied the complex between KIT and imatinib (see Table 1), the hydrogen bond Gly676–Cys673 is altogether absent in the apo form of the kinase. However, the binding of imatinib partially induces the bond (creates a dehydron with a total ρ-value of 19, with three units of ρ provided by the drug). The addition of the extra methyl in WBZ_4 increases this to ρ = 20, thus completely removing the dehydron. As a consequence, the affinity of the drug increases and the inhibition of KIT kinase by WBZ_4 is 23% greater than that of imatinib [38]. This gain is produced by completing the previous partial PIM event (removing the dehydron created during the allosteric binding process). We mention that WBZ_4 was re-engineered on the basis of the complex of imatinib with KIT and was designed as a direct-wrapping event within the complex [38] and not from the apo form as the usual practice in wrapping studies. Because the corresponding hydrogen bond is not present in the apo of the kinase (it is absent in the PDB structure) although it constitutes a dehydron in the complex, this corresponds to a PIM event. This is indeed one of the hydrogen bonds we have already pointed out when we evaluated the potential for incorporating additional wrapper groups to parts of the ligand ion Table 1. The results for the complex between KIT and WBZ_4 are presented in Table 10. In this case, we modified the drug directly in the PDB structure of KIT with imatinib. However, it is worth mentioning that in the re-engineering of the drug [38] computer simulations detected the induced folding formation of the corresponding hydrogen bond, which was altogether absent in the apo form of the kinase.

Table 10. Study of the complex of WBZ_4 with KIT. The carbon atom of the methyl added in the re-engineering of the drug (C30) is indicated in bold.

	Apo enzyme	Inhibited enzyme		
Hydrogen bond	ρ	ρ without ligand	ρ with ligand	Wrappers in ligand
Leu595–Val603	20	**19**	22	C1 C6 C20
Gly596–Val603	**18**	21	22	C20
Val603–Gly596	**18**	21	22	C20
Val604–Val622	30	26	29	C19 C18 C20
Ala621–Thr670	32	27	31	C5 C19 C18 C20
Val622–Val604	30	26	29	C19 C18 C20
Lys623–Val668	24	25	29	C19 C18 C17 C20
Glu640–Ala636	**19**	**17**	**19**	C28 C29
Val643–Ser639	24	22	23	C28
Leu644–Glu640	23	**19**	22	C27 C28 C29
Leu647–Val643	27	27	28	C28
Gly648–Leu644	21	**16**	**19**	C27 C28 C29
Val654–Ile808	26	23	25	C15 C25
Leu657–Ile669	28	29	32	C19 C18 C20
Gly658–Ile669	*	31	34	C19 C18 C20
Val668–Lys623	24	25	29	C19 C18 C17 C20
Ile669–Gly658	*	31	34	C19 C18 C20
Thr670–Ala621	32	27	31	C5 C19 C18 C20
Tyr 675–Leu800	25	24	25	**C30**
Gly676–Cys673	*	**16**	20	C1 C6 C5 **C30**
Phe681–Asp 677	21	23	25	C1 C6
Leu800–Gly676	*	20	24	C1 C6 C5 **C30**
Ile808–Asn652	*	23	24	C25
Cys809–Asn797	23	**19**	25	C15 C23 C25 C26 C27 C29
	PDB code 1PKG	PDB code 1T46[a]		

[a] Imatinib modified *in silico*.

Bold values indicate HBs with ρ number below the dehydron threshold (ρ < 20); Shaded rows indicate the existence of a PIM event. *Indicate that the Hydrogen bond is not formed in the corresponding PDB structure.

The second case is the re-engineering of imatinib into WBZ_7 to overcome the imatinib-resistance that arises by means of a mutation in KIT [39]. WBZ_7 is produced by adding a methyl group at carbon C11 in imatinib. The mutation D816V in KIT brings new wrapper groups in the neighborhood of the A814 and F811 residues (valine is common in carbonaceous wrapper groups, whereas aspartic acid is not), thus inducing a dehydron ($\rho = 17$ for the induced hydrogen bond A814–F811) in the complex with imatinib. This hydrogen bond is absent in the apo kinase conformation (it is not present in the PDB structure). However, as can be noted from direct inspection of Table 11, although imatinib is not able to wrap this dehydron, WBZ_7 does wrap it, thus creating a new PIM event which significantly increases the affinity for the mutated kinase. Again, the redesign of imatinib into WBZ_7 was performed by means of a direct-wrapping event within the complex [39]. Because the hydrogen bond is absent in the apo form of the kinase, it corresponded to a PIM event. In our calculations, we assume the coordinates of the complex correspond with that with imatinib, incorporating the added methyl, although the simulations performed during re-engineering of the drug [39] had confirmed formation of the induced hydrogen bond.

To summarize, although the existence of different drug sizes and of additional interactions prevent a quantitative definition of some kind of PIM-efficiency metric, it is clear that additional PIM events produced by small changes in re-engineered drugs do, in fact, increase the binding affinity. The cases studied suggest the practical potential of these techniques.

Conclusions

This work provides a structural interpretation of allosteric modulation of kinase activity based on induced folding. We demonstrate that the induced folded state of the target kinase is actually metastable, more specifically, it is a locally defective structure in which the backbone is incompletely shielded from water attack. This metastable state is different from the more stable apo form. However, the metastable state detected within the protein–ligand complex behaves as an affinity enhancer because the ligand stabilizes the induced defective fold in a productive way by adding protection to the protein backbone. In contrast with previous studies that highlight the importance of wrapping interactions in protein–ligand associations [1–8], we show that a partial induced destabilization of the protein structure is actually required for the ligand to become an effective binder. This finding heralds a novel mode of structural adaptation generically termed productive induced metastability (PIM), be it the induced–degradation–stabilization or the induced–defect–stabilization binding mode. The result may appear counterintuitive because it makes degradation of the apo structure compulsory. However, the paradox is removed once we take into account that a metastable state can be inherently sticky because of the added stabilization brought about by the association with the ligand. The dynamics and molecular design implications of this discovery will be explored in forthcoming work.

Materials and methods

We employed a geometric criterion to detect hydrogen bonds. We considered a hydrogen bond when the nitrogen (N) bonded to the α carbon of a residue and the oxygen (O) of the carbonyl bonded to the α carbon of another residue are < 3.5 Å, and the (minimum) angle between H···O and H–N (H is the hydrogen bonded to N) is $> 140°$. For each hydrogen bond, we calculate its wrapping value, that is, the level of protection from water attack as given by the number of neighboring nonpolar groups, that is, the number of surrounding shielding nonpolar groups [1–8]. Thus, to compute wrapping we need to classify pairwise electrostatic interactions, hydrogen bonds, in terms of a density distribution $P(\rho)$, where ρ is the number of wrapping groups associated with an interaction. Hence, the extent of hydrogen-bond protection (wrapping) can be determined straightforwardly from atomic coordinates (calculated directly from the PDB 3D structure of the apo form of the protein kinase or the kinase–ligand complex of interest). We analyzed the wrapping of different kinases both in complex with allosteric modulators and in the free uncomplexed state (apo form). In the apo form, the wrapping to the backbone hydrogen bonds is intramolecular, engaging the side chains of neighboring residues. Additional wrapping is provided in the complexes by the nonpolar moieties/groups of the binding ligand that surround preformed hydrogen bonds upon association (intermolecular wrapping). Explicitly, the value of ρ of a hydrogen bond (the wrapping of the hydrogen bond [1–8]) is given by the number of neighboring side chain nonpolar carbonaceous groups (CH_n, $n = 0, 1, 2, 3$). When considering the complex with the allosteric ligand, the ρ-counting includes the neighboring carbonaceous groups furnished by the ligand upon association. To define 'neighboring', we introduce the desolvation domain of a hydrogen bond, materialized as the reunion of two intersecting spheres of fixed radius (approximately the thickness of three water layers) centered at the α carbons of the residues paired by the hydrogen bond and count the number of nonpolar groups within the desolvation domain. In structures of PDB-reported soluble proteins, hydrogen bonds are protected on average by

Table 11. Study of the complex between mutated KIT (D816V) and imatinib and the complex between KIT D816V and WBZ_7. In the second case, the C atom added in the re-engineering of the drug (C31) is indicated in bold.

Hydrogen bond	Apo enzyme ρ	Imatinib ρ without ligand	Imatinib ρ with ligand	Wrappers in ligand
Leu595–Val603	20	**19**	22	C1 C6 C20
Gly596–Val603	**18**	21	22	C20
Val603–Gly596	**18**	21	22	C20
Val604–Val622	30	26	29	C19 C18 C20
Ala621–Thr670	32	27	31	C5 C19 C18 C20
Val622–Val604	30	26	29	C19 C18 C20
Lys623–Val668	24	25	29	C19 C18 C17 C20
Glu640–Ala636	**19**	**17**	**19**	C28 C29
Val643–Ser639	24	22	23	C28
Leu644–Glu640	23	**19**	22	C27 C28 C29
Leu647–Val643	27	27	28	C28
Gly648–Leu644	21	**16**	**19**	C27 C28 C29
Val654–Ile808	26	23	25	C15 C25
Leu657–Ile669	28	29	32	C19 C18 C20
Gly658–Ile669	*	31	34	C19 C18 C20
Val668–Lys623	24	25	29	C19 C18 C17 C20
Ile669–Gly658	*	31	34	C19 C18 C20
Thr670–Ala621	32	27	31	C5 C19 C18 C20
Gly676–Cys673	*	**16**	**19**	C1 C6 C5
Phe681–Asp 677	21	23	25	C1 C6
Leu800–Gly676	*	20	23	C1 C6 C5
Ile808–Asn652	*	23	24	C25
Cys809–Asn797	23	**19**	25	C15 C23 C25 C26 C27 C29
	PDB code 1PKG D816V[a]	PDB code 1T46 D816V[a]		

Hydrogen bond	Apo enzyme ρ	WBZ_7 ρ without ligand	WBZ_7 ρ with ligand	Wrappers in ligand
Leu595–Val603	20	**19**	23	C1 C6 C20 **C31**
Gly596–Val603	**18**	21	23	C20 **C31**
Val603–Gly596	**18**	21	23	C20 **C31**
Val604–Val622	30	26	29	C19 C18 C20
Ala621–Thr670	32	27	31	C5 C19 C18 C20
Val622–Val604	30	26	29	C19 C18 C20
Lys623–Val668	24	25	29	C19 C18 C17 C20
Glu640–Ala636	**19**	**17**	**19**	C28 C29
Val643–Ser639	24	22	23	C28
Leu644–Glu640	23	**19**	22	C27 C28 C29
Leu647–Val643	27	27	28	C28
Gly648–Leu644	21	**16**	**19**	C27 C28 C29
Val654–Ile808	26	23	25	C15 C25
Leu657–Ile669	28	29	32	C19 C18 C20
Gly658–Ile669	[a]	31	34	C19 C18 C20
Val668–Lys623	24	25	29	C19 C18 C17 C20
Ile669–Gly658	*	31	34	C19 C18 C20
Thr670–Ala621	32	27	31	C5 C19 C18 C20
Gly676–Cys673	*	**16**	**19**	C1 C6 C5
Phe681–Asp 677	21	23	25	C1 C6
Leu800–Gly676	*	20	23	C1 C6 C5
Ile808–Asn652	*	23	24	C25
Cys809–Asn797	23	**19**	25	C15 C23 C25 C26 C27 C29
Ala814–Phe811	*	**17**	**18**	**C31**
	PDB code 1PKG D816V[a]	PDB code 1T46 D816V[a]		

[a] Enzymes mutated *in silico*, imatinib modified *in silico*.

Bold values indicate HBs with ρ number below the dehydron threshold (ρ < 20); Shaded rows indicate the existence of a PIM event. *Indicate that the Hydrogen bond is not formed in the corresponding PDB structure.

$\rho = 26.6 \pm 7.5$ side chain nonpolar groups for a desolvation sphere of radius 6 Å [1–8]. Thus, structural deficiencies lie in the tail of the ρ-distribution, i.e. their microenvironment contains 19 or fewer nonpolar groups, therefore their ρ-value is below the mean (= 26.6) minus one standard deviation (= 7.5). Although the statistics on ρ-values for hydrogen bonds vary with the radius, the tails of the distribution remain invariant, thus enabling robust identification of structural deficiencies [1–8]. Such underprotected interactions have been named dehydrons [1–8], a structural motif that has been extensively discussed in the literature and identified in soluble proteins with PDB-reported structure [1–8]. As in previous work [7,8], we only considered backbone hydrogen bonds and decided to leave aside side chain–side chain hydrogen bonds from our analysis based on the following grounds. The fluctuational nature of surface side chains imposes an entropic cost associated with hydrogen bond formation which makes the latter marginally stable at best. Also, the wrapping statistics for side chain hydrogen bonds are essentially flat with no clear distinction of the tails of the distribution due to the conformational richness of the side chains.

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Author contributions

Conceived and designed the research: GAA, AF. Performed the research: JMM-O, JA R-F. Analyzed the data: JMM-O, JA R-F, GAA, AF. Contributed analysis/tools: JMM-O, JA R-F. Wrote the paper: GAA, AF.

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