Dynamical network of residue–residue contacts reveals coupled allosteric effects in recognition, catalysis, and mutation

Urmi Doshi^a, Michael J. Holliday^b, Elan Z. Eisenmesser^b, and Donald Hamelberg^{a,1}

^aDepartment of Chemistry, Georgia State University, Atlanta, GA 30302; and ^bDepartment of Biochemistry and Molecular Genetics, University of Colorado Denver, Aurora, CO 80045

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Detailed understanding of how conformational dynamics orchestrates function in allosteric regulation of recognition and catalysis remains ambiguous. Here, we simulate CypA using multiple-microsecond-long atomistic molecular dynamics in explicit solvent and carry out NMR experiments. We analyze a large amount of time-dependent multidimensional data with a coarse-grained approach and map key dynamical features within individual macrostates by defining dynamics in terms of residue–residue contacts. The effects of substrate binding are observed to be largely sensed at a location over 15 Å from the active site, implying its importance in allostery. Using NMR experiments, we confirm that a dynamic cluster of residues in this distal region is directly coupled to the active site. Furthermore, the dynamical network of interresidue contacts is found to be coupled and temporally dispersed, ranging over 4 to 5 orders of magnitude. Finally, using network centrality measures we demonstrate the changes in the communication network, connectivity, and influence of CypA residues upon substrate binding, mutation, and during catalysis. We identify key residues that potentially act as a bottleneck in the communication flow through the distinct regions in CypA and, therefore, as targets for future mutational studies. Mapping these dynamical features and the coupling of dynamics to function has crucial ramifications in understanding allosteric regulation in enzymes and proteins, in general.

allostery | enzyme dynamics | residue–residue contacts | Cyclophilin A | molecular dynamics

As a biomolecule samples various conformations governed by its free energy landscape, it undergoes a wide spectrum of motions that range over a broad timescale and length scale (1). Except for certain cases in which large-scale displacements are observed upon ligand binding (2), these motions, in general, account for modest fluctuations around an average native structure (3). Despite extensive studies, detailed understanding of how conformational dynamics lead to or facilitate function remains formidable (4–6). It has been frequently observed that enzymes in their free unliganded state sample 3D conformations that consist of those visited in the presence of the ligand (7). Differences in intrinsic conformational dynamics in the wild type and the mutant maltose-binding proteins have been shown to be related to association and dissociation of ligand and thereby to affect dissociation constants (8). Enzyme dynamics in Cyclophilin A (CypA), a peptidyl prolyl *cis*–*trans* isomerase, have been demonstrated to occur on the same millisecond timescale as the catalytic turnover (9). Mutants of dihydrofolate reductase that lead to suppressed conformational dynamics in the active site have exhibited concomitant loss in enzymatic activity (10).

These exemplary studies are suggestive of a key role for conformational dynamics in recognition and catalysis. This notion has been controversial (10–13) due to limited or, in some cases, a complete lack of microscopic analysis of experimental observations. The suppressed catalytic activity in mutants may be a result of an increase in activation energy and not decreased dynamics (14, 15). Although enzyme dynamics may not be responsible for catalytic speed up relative to the uncatalyzed reaction, CypA dynamics has been shown to be coupled to catalytic function (16). Allosteric

regulation, i.e., modification of binding or catalysis at the active site due to binding of a ligand at a distal nonoverlapping site, is widespread in biochemical signaling (17, 18). It is natural that allosteric regulation depends on modulation of protein motions, because substrate binding and catalysis are linked to conformational dynamics (19, 20). Unlike static X-ray structures, solution NMR relaxation dispersion techniques have been instrumental in providing high-resolution conformational exchange information using site-specific isotope labeling (21). In NMR studies of CypA, a dynamic continuum has been identified such that the relaxation profiles cannot be globally fit to one or two exchange phenomena and are instead indicative of more localized motions (22). Exchange rates coalesce somewhat during turnover, perhaps suggesting an increase in coordination throughout the protein, but appear to still consist of localized motions that are not fully coherent (23). However, dynamical signals during catalytic turnover could be affected by substrate binding and unbinding, especially if the substrate binding affinity is low, thereby leading to ambiguity in the interpretation of NMR analysis (23).

Complementary to experiments, long-timescale molecular dynamics (MD) simulations can be greatly instrumental in providing a microscopic picture of biomolecular dynamics and establishing its exact linkage to function. However, the challenge at hand is to elucidate the key dynamical features and correlations between different parts of the protein from a vast amount of multidimensional time-dependent data from MD. Principal component analysis (PCA) is often used to reduce the dimensionality of conformational space and map the differences in conformational

Significance

How exactly protein motions facilitate substrate recognition and catalysis has remained largely unanswered. Characterization of protein dynamics at atomistic level is essential to understanding function. Molecular dynamics and NMR are helpful in this regard; however, analyzing multidimensional data from very long molecular dynamics (MD) simulations to elucidate key dynamical features observed in NMR remains very challenging. We present results from an approach for data analysis in which dynamics is defined in terms of interresidue contact formation and breaking. Analyzing simulation data on a therapeutically important Cyclophilin A and carrying out NMR experiments, we uncovered remarkable and unprecedented changes in its motions at a site over 15 Å from the active site upon substrate binding and how mutation in this distal site affects catalysis.

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¹To whom correspondence should be addressed. Email: dhamelberg@gsu.edu.

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ensembles. PCA, which is usually performed on Cartesian coordinates, may sometimes mask certain important long-range dynamical relations and complex features of biomolecular conformational dynamics. Comparing residue–residue contacts in various isoforms from difference contact maps built from simulation trajectories has aided in determining certain similar structural properties and some unique to a particular isoform (24). Another analysis that involves monitoring the time evolution of residue–residue contact formation and breaking has been proven useful in identifying certain characteristic events during conformational transitions (25). Correlated motions between different biomolecular segments have been identified using cross-correlation analysis and building dynamical networks (25–29). However, interpretation of the results of such analysis in case of CypA, which exhibit subtle changes upon substrate binding and catalysis, has been ambiguous.

Here, we characterize the conformational dynamics of CypA using very long atomistic standard MD simulations in explicit solvent, and the results are validated using NMR experiments. CypA is an archetypal and extensively studied enzyme belonging to the family of peptidyl prolyl isomerases (PPIases), speeding the *cis*–*trans* isomerization of peptidyl prolyl ω -bond in its protein substrates by more than 10^5 times (30–34). In five independent microsecond-long simulations, we monitored the dynamics in wild-type CypA, V29L variant of CypA, CypA bound to a substrate analogue in the *trans*, transition state, and *cis* configurations. When we apply our method of analyzing the trajectories at a coarse-grained level, i.e., inter-residue contact interactions, and use PCA in contact space, the specific differences in CypA dynamics upon association to its substrate, during the catalytic process and upon alteration of a single residue distant from the active site are revealed.

Results and Discussion

Analyzing Standard Molecular Dynamics Trajectories at the Residue-Level Interactions. We analyzed the trajectories of the free wild-type CypA (C_{wt}) and CypA in complex with the substrate (C_{trans} , C_{ts} , and C_{cis}) to reveal the differences in the conformational ensembles of the free and substrate-bound enzyme and map the dynamical features in each ensemble (Figs. S1A and S2). Mining the time evolution data of 3N atomic coordinates ($n = 2,505$ atoms of CypA) for key features presents a “big data” problem. To simplify the complexity of the high-dimensional data, we established a data reduction scheme similar to the Computation of Allosteric Mechanism by Evaluating Residue–Residue Associations approach (35). The 3D Cartesian coordinates of the trajectory is condensed to a binary trajectory (consisting of 0s and 1s) of $N_{res} \times (N_{res}-1)/2$ residue–residue contacts. A contact is defined to have formed between two residues if the distance between any of their heavy atoms is within a cutoff of 4.5 Å during the simulation. It is common to use such cutoff values in coarse-grained representation of protein structures (36–38) and evaluation of protein contacts (39, 40). A contact receives a value of either 1 when formed or 0 when the contact is broken. Although some contacts that are essential for the structural integrity of CypA will be present throughout the simulation and have an f value near 1.0, others may never be formed (f close to 0) (Fig. 1). The region around the transition midpoint of the sigmoidal contact probability curve represents those contacts that are formed and broken as time evolves as a consequence of conformational dynamics. These interesting contacts are potentially relevant for ligand recognition, allosteric communication, conformational transitions, and function, as they can form and break and are easily perturbed upon ligand binding or during catalysis. From the average contact probability curve (Fig. 1B, Inset), we selected the contacts in the transition region between 10% and 90% for further analysis (Fig. 1 and Fig. S1). This cutoff was decided based on an error analysis between the first and the second half of the simulation (Fig. S3). We refer to these contacts as dynamic contacts (c_{dyn}) and the residues that are involved in formation of these contacts as dynamic residues (r_{dyn}). As these contacts represented less than 2% of the unique interresidue

Fig. 1. Structure of CypA and the dynamical network of residue–residue contacts in CypA. (A) The selected contacts (shaded area in B) are represented as cylinders connecting the residue pairs on the CypA structure. (B) Residue–residue contacts within CypA are ranked according to their probability of formation, f (i.e., fraction of the simulation time when a contact is formed). Contact probabilities are plotted against their respective ranked contact index. This curve represents the average from individual simulations of free C_{wt} , C_{trans} , C_{ts} , and C_{cis} . Inset shows a magnified portion of the curve with the shaded area depicting contacts that are formed between 10% and 90% of the simulation time.

contacts, not only the size of the binary trajectory but also the noise in the multidimensional data were drastically reduced.

Validating our Coarse-Grained Approach to Analysis with NMR Chemical Shift Data. We identified 40 residues that exhibited significant NMR chemical shift changes in either their backbone amide or side-chain C_β atoms or both after association with the substrate (Fig. 2A and B). For the contacts selected according to Fig. 1B, we computed the difference in contact probabilities obtained from simulations of the free and substrate-bound CypA. Out of 40 residues, we observed 30 residues that belonged to r_{dyn} (i.e., involved in forming one or more contacts that exhibited more than 10% changes in contact probabilities) in simulations of CypA-substrate complexes (Fig. 2C). From the remaining 10 residues that showed no changes upon substrate binding in our simulations, the immediate neighbors of 8 residues were found to be in r_{dyn} when the substrate was present in the active site. These additional residues were most likely affected by the change in chemical environment, rather than the dynamics, upon binding the substrate. It should be noted that a longer peptide substrate, Gly-Ser-Phe-Gly-Pro-Asp-Leu-Arg-Ala-Gly-Asp, was used in NMR experiments (41) than the one used in the simulations. Majority of the residues that undergo chemical shift changes in the presence of the substrate were observed in our simulations to be perturbed due to dynamical changes and not due to direct interaction with the substrate. Such comparisons between our simulations and chemical shift data thus validated the conformational ensembles obtained from our microsecond-long standard MD simulations as well as our coarse-graining approach to analyzing the trajectories.

Mapping the Dynamical Changes in CypA upon Substrate Binding and Along the Reaction Pathway. NMR chemical shift changes in a protein upon association of a ligand report on the changes in the chemical environment of its residues. It is hard to decipher whether these chemical shift changes are due to ligand binding or the dynamical changes associated with ligand binding. However, whether these active site residues also undergo changes in their dynamics cannot be determined from ensemble-averaged chemical shift data. This issue can be easily addressed from MD trajectories of the free CypA and substrate-bound CypA. We computed the difference in the probabilities of contact formation of c_{dyn} between the ensembles obtained from MD simulations of the free CypA and CypA bound to the *cis* substrate (Fig. 3A). A value of $f_{cis} - f_{free}$ close to +1.0 represents a contact that is formed in the *cis*-bound CypA but rarely or never formed in the free enzyme. A value close to –1.0

Fig. 2. Experimental validation of dynamical change in CypA upon substrate binding. (A) Normalized changes in chemical shift in amide atoms $\sqrt{(\Delta\delta H \times 0.2)^2 + (\Delta\delta N)^2}$ (green) and (B) chemical shift changes in C_β atoms (red) of CypA upon binding the substrate, as obtained from NMR experiments, as previously described (41). (C) Residues that exhibit chemical shift changes of greater than 0.25 ppm and are involved in contacts that show >10% variation in the probabilities of formation (and are a subset of contacts with f between 10% and 90%, i.e., c_{dyn}) upon substrate binding are shown as spheres (C_α atoms) on the CypA structure. The color code is consistent with A and B. Blue spheres represent those residues that are involved in formation of those contacts that have >10% change in their probability of formation upon substrate binding and with chemical shift changes of greater than 0.25 ppm in both the amide and C_β atoms.

signifies a contact formed with higher probability in the free CypA and broken when the *cis* substrate is present in the active site. Mapping these contacts that are formed and broken with varied probabilities upon substrate binding on the CypA structure revealed the involved residues that were predominantly clustered (termed “dynamic cluster”) in a region distinct from the active site (Fig. 3C). Notably, the active site residues were involved in very few contacts that were formed and broken at low probabilities and through which they communicated with the distant cluster of dynamic residues. Indeed most of these dynamic cluster residues also exhibited NMR chemical shift changes, indicating the long-range effects of substrate binding.

Comparison of the contact probabilities of c_{dyn} in the *cis*- and transition-state-bound ensembles of CypA (Fig. 3 B and D) revealed the enzyme residues that exhibited dynamical changes during catalysis. The differences in the contact formation probabilities were relatively less drastic compared with the differences observed upon substrate binding. However, the contacts that are formed and broken in the ensemble of transition-state-bound CypA were distributed throughout the protein. We further performed principal component analysis in contact space (i.e., c_{dyn}) by combining the binary trajectories of C_{wt} , C_{trans} , C_{ts} , and C_{cis} . Although the conformational ensemble of the free CypA was primarily distinct from those of substrate-bound CypA, there was significant overlap between conformational ensembles of CypA bound to the substrate in different configurations along the reaction pathway. This indicated that overall a new set of contacts were formed and broken upon substrate association. We would like to note that PCA performed on contact space reports on features that are distinct from PCA of fluctuations in Cartesian coordinates (16, 42). PCA in contact space defines conformational ensemble in terms of contacts formed and broken and thus yields more information of dynamic changes that take place upon substrate binding or catalysis.

We further computed the average time it took to form and break each c_{dyn} contact from the trajectories of C_{wt} , C_{trans} , C_{ts} , and C_{cis} . The time constants (τ) exhibited an overall distribution ranging from picoseconds to microseconds (Fig. S4) that altered upon substrate binding (i.e., comparing Fig. S4A with Fig. S4 B–D) and when the

bound substrate was in a different configuration (i.e., comparing Fig. S4 B, C, and D). Interestingly, contacts with slower dynamics than the picosecond timescale were primarily clustered in the region that was distant from the active site. It was only in the C_{ts} that a higher number of active site residues were involved in the formation and breakage of contacts on a relatively slower timescale. Furthermore, certain contacts that took more than 100 ns to form and break in C_{wt} , C_{trans} , and C_{cis} were completely absent when the substrate was in the transition-state configuration, thereby narrowing the range of contact dynamics timescale in C_{ts} . These results suggested non-coherent contact dynamics that was altered upon association of the substrate and during the various stages of catalysis.

Effects of a Distal Mutation within the Dynamic Cluster of Residues on CypA Catalysis. Interestingly, it was observed in previous NMR studies by Schlegel et al. that Valine 29, which is part of the dynamic cluster, exhibited significant variation in dynamical motion upon substrate binding to CypA compared with unbound CypA (22). Due to the observed dynamic coupling between the active site and the dynamic cluster from the previous NMR studies, we speculated that the effects of mutating Valine 29 in this distal region with a residue that did not significantly perturb the dynamics and maintained the fold and integrity of CypA should be transmitted back to the active site of CypA. The question was whether a mutation in the dynamic cluster that was over 15 Å away from the active site would exert any effect on the active site residues or the activity of CypA. We carried out a conservative mutation within this region by mutating Valine 29 (Fig. 4A) to Leucine and performed NMR studies to determine the effects of the mutation on the catalytic isomerization rate of CypA. The isomerization rates were determined by NMR ZZ exchange, as previously described (41) (Fig. 4 B and C). This single conserved mutation resulted in a decrease in the catalytic isomerization rate from $10.4 \pm 0.4 \text{ s}^{-1}$ for the wild-type CypA to $6.9 \pm 0.3 \text{ s}^{-1}$ for the V29L mutant i.e., the V29L mutant CypA catalyzed at ~70% the rate of wild-type CypA. These results provided a direct test of the significance of the cluster of residues that are perturbed on substrate binding and further raised the question as to how the effects of modification of a single residue distant from the active site are communicated to the active site.

We simulated V29L CypA ($C_{mutV29L}$) for 2.2 μs with standard MD to address this question. Similarly, we selected the set of contacts (C_{dyn}) with probability of formation between 10% and 90% from an average ranked contact probability curve (computed from the ensembles of C_{wt} and $C_{mutV29L}$; Fig. S5A). Comparing the contact trajectories of C_{wt} and $C_{mutV29L}$ showed that most of the contacts that were formed and broken with probability of >10% were expectedly found in the region around Val29 (Fig. 4 D and E). However, the cluster of contacts around V29 was dynamically connected or coupled to the active site through few contacts that were formed and broken with relatively lower probabilities upon mutation (Fig. 4E).

Structural studies have demonstrated that a conserved active site residue, Arg55, is well positioned to stabilize the transition state of the substrate and thereby facilitates catalysis (43). Mutating Arg55 to Ala or Lys has been shown to result in drastic loss of catalytic efficiency (7, 44). Previous quantum mechanics/molecular mechanics (QM/MM) (45) and MD studies (46, 47) have shown that Arg55 is involved in key stabilizing hydrogen bonds with the substrate in the transition state. Given the importance of Arg55 in binding and catalysis, we monitored its side chain motion by computing the distance between the guanidinium carbon of the side chain of Arg55 and backbone C_α of His126 (Figs. 4A and S6). In the C_{ts} ensemble, the predominantly unimodal probability distribution of this distance peaked at 13.5 Å (Fig. S6), indicating a relatively fixed and less dynamic backbone at the active site’s key residue. In C_{wt} , however, the distribution exhibited a second peak centered on ~15.5 Å that constituted the major population (Figs. 4H and S6). Such a multimodal distribution suggested the motions of the side chain of Arg55, moving away and approaching the active site and

Fig. 3. Dynamical changes in CypA following substrate binding and during catalytic function. (A) Differences in the fraction of the time the selected contacts (c_{dyn}) are formed in trajectories of free CypA and CypA bound to the substrate in the *cis* configuration. Positive (blue) and negative (red) values signify contacts that are formed and broken, respectively, in CypA upon binding the *cis* substrate. (B) Differences in the fraction of the time the selected contacts are formed in trajectories of CypA bound to substrate in the transition state and the *cis* state. Positive values signify contacts that have higher probability of forming in the ensemble of CypA structures when bound to the substrate in the transition state. Negative values signify contacts that have higher probability of forming in CypA when bound to the *cis* substrate. (C) For CypA contacts that show a difference of more than 10% in the probability of contact formation or breaking upon substrate binding [i.e., $\text{abs}(f_{cis} - f_{free}) \geq 0.1$] are shown as blue and red cylinders, respectively, on the CypA structure (gray). The cylinders are shown to join the C_{α} atoms of the pair of residues. The radius of the cylinder is proportional to the absolute values of $f_{cis} - f_{free}$. (D) CypA (gray) with red and blue cylinders showing contacts broken and formed, according to the criterion mentioned in C. The radii of the cylinders are proportional to the absolute values of $f_{ts} - f_{cis}$. (E) Projection of contact space on the first two principal components— c_{dyn} contacts that are formed and broken in each snapshot of the trajectories of C_{wt} (black), C_{trans} (blue), and C_{ts} (green), and C_{cis} (red) are shown.

substrate. Interestingly, in the V29L mutant, there was a redistribution such that the major population was shifted to 13.5 Å (Fig. 4H), reminiscent of the well-organized and less dynamic active site in the transition-state-bound CypA (C_{ts} ; Fig. S6). These results suggested that the active site of the V29L mutant is preorganized—a characteristic that might confer slightly higher substrate binding affinity to V29L mutant over the wild-type CypA. As C_{ts} binds better than C_{trans} or C_{cis} , the V29L mutant is expected to bind the substrate better as well. In CypA, *trans* and *cis* states are both substrates and products, because CypA catalyzes *cis*–*trans* isomerization reversibly. Assuming the mutant CypA interacts with the transition state in a manner similar to the wild type, the increased binding affinity would confer

relatively decreased transition-state stabilization by the mutant CypA than the wild-type CypA and thereby relatively increase the activation barrier for catalysis. Similarly, it has been shown that substrates of CypA with optimal binding do not necessarily have the highest catalytic rates (43). Also, substrates that bind optimally may not dissociate from the enzyme with the same ease as those with lower binding affinity, thereby resulting in lower catalytic efficiency. Therefore, relatively stronger binding of the substrate is not always ideal for catalysis.

We further projected the configurations from each snapshot on to the two most dominant principal components obtained from PCA of C_{wt} and $C_{mutV29L}$ contact trajectories (Fig. 4I). The two ensembles were remarkably well separated in contact space,

Fig. 4. Dynamical changes in CypA upon mutation. (A) On the CypA structure are shown the C_{α} atoms of the mutated residue Val29 (red sphere) and those of active site residues, Arg55 and His126 (green spheres). Decay of ZZ-exchange peak intensity spectra of residue Leu7 in the presence of (B) wild-type CypA and (C) the V29L mutant. Data points are shown for the *trans* (black) and *cis* (red) peaks, *trans*–to–*cis* (green), and *cis*–to–*trans* (blue) cross-peaks. Continuous lines are best fits to the data. Representative ZZ-exchange spectra collected on ^{15}N -labeled substrate at 144 ms in the presence of wild-type CypA and the V29L mutant are shown as *Inset* to B and C, respectively. Arrows indicate the exchange peaks in the presence of the enzyme. (D) Differences in the fraction of the time the selected contacts (c_{dyn}) are formed in trajectories of wild-type CypA and V29L mutant of CypA. Positive (blue) and negative (red) values signify contacts that are formed and broken, respectively, with higher probabilities in the mutant than the wild type. (E) Contacts having an absolute difference in contact probabilities between the wild-type and the mutant (i.e., $f_{mutV29L} - f_{wt}$) > 0.1 are shown as cylinders connecting the C_{α} atoms of residues with the same color code as in D. The radii of the cylinders are proportional to the $\text{abs}(f_{mutV29L} - f_{wt})$. The selected contacts from the trajectories of (F) wild-type CypA and (G) V29L mutant are color coded according to their time constants (τ) of formation and breakage: $\tau < 0.1$ ns (white), $0.1 \text{ ns} \leq \tau < 1$ ns (red), $1 \text{ ns} \leq \tau < 10$ ns (orange), $10 \text{ ns} \leq \tau < 100$ ns (green), and $\tau \geq 100$ ns (blue). The radii of the cylinders between the C_{α} atoms are proportional to the magnitude of τ . (H) Probability distribution of the distance between the guanidinium carbon of the side chain of Arg55 and backbone C_{α} atom of His126 in the wild-type (black) and the V29L mutant CypA (orange). (I) Projection of contact space on to the first two principal components. Each data point represents a configuration of either the wild-type (black) CypA or its mutant (orange) in contact space.

in information passage between distant residues. We found that contact dynamics is dispersed over a timescale that ranges from picoseconds to longer than hundreds of nanoseconds. Our results suggest that these dynamical features have crucial importance in the allosteric regulation of biological systems by revealing the coupling of protein dynamics to function. Mapping these dynamical features has ramifications in fully understanding the relationship between dynamics and function in biomolecular systems.

Materials and Methods

Molecular Dynamics Simulations. For each system—C_{wt}, C_{trans}, C_{ts}, C_{ts}, and C_{mutV29L}—the initial structures of CypA were solvated in individual TIP3P (48) water boxes. Standard molecular dynamics were carried out for each system at 300 K for a total simulation time of 11 μ s. Operational details on molecular dynamics are included in the [Supporting Information](#). Contact analysis and calculation of centrality measures are described in detail in the [Supporting Information](#).

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NMR. The NMR chemical shifts were determined as previously described (41). The catalytic isomerization rates were determined by ZZ exchange as previously described (41). More specifically, ZZ-exchange data were collected on 1 mM ¹⁵N-labeled peptide with 20 μ M unlabeled wild-type CypA or V29L CypA mutant at 10 °C on a Varian 600 MHz spectrometer with a cryogenically cooled probe. Data were fit according to Farrow et al. (49).

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