Prediction and validation of protein intermediate states from structurally rich ensembles and coarse-grained simulations

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Protein conformational changes are at the heart of cell functions, from signalling to ion transport. However, the transient nature of the intermediates along transition pathways hampers their experimental detection, making the underlying mechanisms elusive. Here we retrieve dynamic information on the actual transition routes from principal component analysis (PCA) of structurally-rich ensembles and, in combination with coarse-grained simulations, explore the conformational landscapes of five well-studied proteins. Modelling them as elastic networks in a hybrid elastic-network Brownian dynamics simulation (eBDIMS), we generate trajectories connecting stable end-states that spontaneously sample the crystallographic motions, predicting the structures of known intermediates along the paths. We also show that the explored non-linear routes can delimit the lowest energy passages between end-states sampled by atomistic molecular dynamics. The integrative methodology presented here provides a powerful framework to extract and expand dynamic pathway information from the Protein Data Bank, as well as to validate sampling methods in general.
Proteins function as sensors that cycle between different states in response to external stimuli. In general, stable conformers captured experimentally represent the end states of the functional cycle, while short-lived or highly flexible intermediates along the transition—which often hold the key to understand molecular mechanisms—are difficult to trap. Although a host of theoretical strategies have been developed to sample transition pathways, the intrinsic difficulty to predict the routes for conformational change and the lack of experimentally resolved intermediates hamper the validation of path-sampling methods.

Hitherto, in silico pathways are typically evaluated on the basis of stereochemical quality of the structures or by tracking progression along system-defined coordinates. However, the selection of heuristic collective variables (CVs) is non-trivial and dimensionality reduction can be problematic. Structural quality or progression along a few order parameters does not assure that a pathway samples biologically relevant routes to connect end-states. An interesting approach, proposed by Weiss and Levitt, is to benchmark path-sampling methods against proteins with at least three distinct states solved, and measure how close the sampled pathway spontaneously approaches known intermediates in terms of root mean square deviation (rMDS). Still, such procedure cannot assess the feasibility of the movements or to what extent they correspond to the biological motions. To address this issue we propose to take a step beyond simple two- or three-state benchmarking by making an ensemble-level analysis that considers all structural information available in the Protein Data Bank (PDB) for a given protein. Although there have been works systematizing protein motions in databases, a general and reliable framework to unlock and expand the pathway information contained in structural ensembles is still missing.

Principal component analysis (PCA) is a powerful technique to decode ensemble motions and has been successfully applied to extract principal components (PCs) from experimental ensembles and to evaluate normal modes (NMs) as well as essential motions obtained from molecular dynamics (MD) simulations. For example, McCammon and co-workers showed the utility of PCs obtained from X-ray structural ensembles as CVs to track MD; a recent work used PCs to estimate free-energies of transitions. Here we build on the idea to use the two dominant PCs as complex multidimensional reaction coordinates to reveal the direction of ensemble-encoded conformational changes. The key to our analysis is a selection criterion different from previous ensemble-based studies, more focused on the quantity rather than the quality of the sampling by experimental structures. We argue that, only when the solved structures (regardless of their number) sample at least three different interconnected conformations, the PCs provide optimal CVs to highlight transition paths in the conformational landscape. By focusing on five structurally rich and diverse model systems we demonstrate that X-ray ensemble PCA accurately clusters resolved structures into different functional states. We show that for these proteins, the representation of the conformational space is robust even with minimal numbers of structures as long as they are well distributed along interconnecting paths. The projection of experimental conformers onto the PC-space provides an excellent visual representation of the structural landscape for a protein with known intermediates. Importantly, it also allows for immediate evaluation of the sampled pathways as it gives information on the natural sequence of on-pathway intermediates, which in turn can reveal information on their functional significance.

On the basis of the proposed ensemble PCA, we compare the performance of a novel coarse-grained (CG) path sampling algorithm named eBDIMS, using elastic network model (ENM) driven Brownian simulations, with several well-established methods as well as with state-of-the-art MD simulations (see Methods). Path sampling algorithms span from simple morphings based on interpolations in Cartesian or internal coordinates, to geometrical targeting or atomistic techniques based on energy minimization. A number of MD-based approaches are applied to explore transitions, for example the nudged elastic band or the ‘strings’ method, as well as enhanced sampling algorithms such as conformational flooding, metadynamics, accelerated MD or the accelerated weight histogram (AWH) method. Although accurate, these techniques are computationally expensive and limited to small systems and short timescale transitions. CG-models, where each residue is reduced to a few beads interacting by simple potentials, minimize computational costs. Among CG-methods, ENMs are conceptually simple but capable of predicting accurately conformational changes. Despite reducing protein architecture to a minimalist network of Cx-carbons connected by springs, NMs computed from the ENM potential describe transitions between X-ray pairs with surprising precision and reproduce the flexibility from experimental ensembles or long MD simulations. For years, ENMs have been at the core of transition methods from simple interpolations to two-state ENMs. Being limited to an equilibrium basin, pathway generation requires iterative computation and deformation along selected NMs, which can produce stereochemical distortions. Although these issues can be reduced applying internal coordinates, or just using the modes to bias more realistic simulations, the mode selection still poses a problem. Here the use of the network potential in the context of a BD simulation avoids unrealistic structure deformations and provides spontaneous sampling along the relevant modes. We show that, compared with other approaches, eBDIMS smoothly samples the experimentally encoded motions, and can predict the sequence of intermediates as accurately as Climber, an atomistic method based on the Energy Calculation and Dynamics (ENCAD) molecular-mechanics force-field, but with the versatility of a simulation.

The integrated analysis of the PCs and the in silico pathways provides novel insights into the conformational changes of the studied proteins. We further demonstrate that simple algorithms such as eBDIMS or Climber accurately sample the conformational space given by experimental data and MD, predicting the lowest energy paths defined by transition intermediates. In conclusion, the methodology outlined here provides a powerful framework to extract and expand dynamic information from the rapidly growing PDB to evaluate sampling methods or even the functional status of new experimental structures.
for PCA; all analyses focused on the two major PCs, covering >70% of the variance (Supplementary Table 2) and 95% of the transitions (Supplementary Table 6). For non-linear methods, trajectories were computed in forward/reverse directions, and eBDIMS pathway asymmetry quantified (see Methods). Finally, eBDIMS trajectories were compared with the free energy landscapes (FELs) sampled by MD for three representative examples. Interactive PC1-2 plots are provided in Supplementary Data 1. An alternative version can be found at http://data.tcblab.org/doi/10.1038/ncomms12575/Transitions.html.

**Escherichia coli** ribose-binding protein (RBP). RBP is a periplasmic protein that binds ribose with a 6Å hinge motion of two similar domains. Although its crystallographic ensemble only contains eleven structures, they cover the entire closing process; all the conformers exist at equilibrium and ribose concentration shifts their distribution. We selected the open (apo) structure 1BA2 and the closed (bound) 2DRI as the end-states (Fig. 2a). The first PC (PC1), which describes domain closing (bending), accounts for most of the variance (97%) of the transition, while the second PC2 describes a subtle oscillation (twisting; 2%) (Fig. 2b, Supplementary Tables 2 and 6). PC1 broadly separates the ensemble into three clusters of decreasing opening angle (Supplementary Fig. 3a): first, the unbound conformers, then intermediates 1URP and 2GX6, and finally the ligand-bound cluster. There is an excellent alignment of the first NM with the distribution of experimental structures along the path; however, this mode is not the best aligned with the transition vector between the end points (Supplementary Fig. 4a and Supplementary Table 7). Trajectory projection onto the PC1-2 subspace shows that the examined methods differ notably in how they sample the X-ray motions. Although they all approach the existing intermediates (Fig. 2c, right), reaching as close as 0.5–1.5 Å rMDS (Fig. 2d and Supplementary Table 5), the PC1-2 Euclidean distances discriminate between paths that resemble a straight interpolation and the ones that explore the subtle PC2 oscillations that accompany protein closure. Interestingly, eBDIMS and Climber converge in the reverse pathway through a straight-like route with a slightly smaller rotational deviation along PC2. However, the asymmetry score is low (0.1; Supplementary Fig. 5), and as shown by MD, both routes are actually sampling the edges of the same low energy passage connecting the end-conformations (see Supplementary Fig. 6a and FEL below).

**E. coli** 5′-nucleotidase (5′-NTase). 5′-NTase, an enzyme that hydrolyses nucleotides, is formed by two globular domains linked by an α-helix. Upon binding, an unusual 96° ball-and-socket rotation (rMDS 9.3 Å) moves the ligand along the interdomain surface into the catalytic site. The X-ray ensemble (sixteen structures) covers domain closure, with intermediates trapped by Cys-bridges. We selected the open 1OID and closed (RNA-bound) 1HPU structures as end-states (Fig. 3a). Again, PCA decomposes the ensemble into a major PC1 capturing most of the ball-and-socket motion (95%) of the transition (Supplementary Fig. 3b) and a minor PC2 tracking a subtle orthogonal rotation (4%) (Fig. 3b and Supplementary Tables 2 and 6). PC1 alone clusters the structures into three functional groups: the open structures (1OID and others), the intermediates (1O18 and 4WWL), and the catalytically competent closed state (1HPU, 1HOS), while PC2 further helps to rank path sampling algorithms (Fig. 3c). The intermediates are sequentially visited by all methods reaching as close as 1.8 Å rMDS with eBDIMS (Fig. 3d and Supplementary Table 5), but projection onto PC2 reveals instabilities in some of the ENM algorithms. Once again, the NMs of the end-states perfectly align with the distribution of experimental structures (not shown) and the direction of conformational change (Supplementary Table 7). Both eBDIMS and Climber smoothly sample the forward transition, which departs along PC2, while the reverse paths proceed straight-forward. As in RBP, pathway asymmetry is low (0.2; Supplementary Fig. 5), indicating that both routes in fact explore the same low energy through between the end-states, a notion further supported by spontaneous sampling from unbound forms in MD (Supplementary Fig. 6b, see below).

**Aequifex aeolicus** ribonuclease III (RNaseIII). RNaseIII is an Mg²⁺-dependent enzyme that modulates gene expression by
cleaving double-stranded RNA (dsRNA). It is a symmetric homodimer where each subunit is composed of two domains (an RNaseIII domain, RIID, and a dsRNA-binding domain, dsRBD) separated by a flexible linker. RIID dimerization forms a ‘catalytic valley’ to accommodate dsRNA; the dsRBDs need to rotate dramatically (180°) to position RNA on it. However, this region is negatively charged, requiring Mg\(^{2+}\)-coordination to mitigate dsRNA repulsion. In absence of Mg\(^{2+}\), dsRNA binds outside the valley in a ‘non-catalytic’ form (1YYO, 2NUE), but with Mg\(^{2+}\) present, it moves inside the valley leading to a ‘catalytic’ form (2NUG, 2NUF, 2EZ6). Since a true apo structure is missing, the 11 RNA-bound crystallized structures represent intermediate snapshots in dsRNA processing between non-catalytic/catalytic states. Therefore, we focused on the transition between the non-catalytic complex RNaseIII–RNA4 (1YYO), and the pre-catalytic RNaseIII–RNA3 (1YYW), where dsRNA is reorienting (Fig. 4a). Between these two conformers (18 Å rMSD), there is a well-characterized intermediate, RNaseIII E110Q–RNA2 (1YZ9; ref. 49). Here the complexity of the movements decomposes the ensemble into two similarly weighted components (Fig. 4b and Supplementary Table 2): PC1 describes dsRBD-arms opening or ‘breathing’ \(^{49}\) (51% variance), while PC2 tracks their concerted rotation (43% variance) (Supplementary Fig. 3c) capturing most of the transition (see Supplementary Table 6). Altogether they separate the structures into four functional groups: the 1YYO cluster, the intermediate 1YZ9, the pre-catalytic 1YYW cluster and catalytic 2EZ6 cluster (Fig. 4c, left). Along PC1 the structures separate into only three groups according to dsRBD-opening, because the closed non-catalytic and catalytic complexes are not differentiated; PC2 clearly distinguishes their opposite orientations (with the RNA-binding surface looking outwards/inwards to the catalytic valley). Four of the methods (Fig. 4c, right) cannot track this challenging transition, which both eBDIMS and Climber sample smoothly visiting the 1YZ9 intermediate within 4 Å rMSD (Fig. 4d, Supplementary Table 5 and Fig. 6a,b). As above, the lowest NMs point to the nearest intermediate rather than to the transition direction (Supplementary Fig. 4b and Supplementary Table 7); at the 1YYW bifurcation, they split into two directions pointing back to 1YYO or to the Mg\(^{2+}\)-bound region (not shown). Here, the asymmetry score is high (0.4; Supplementary Fig. 5) but the trajectories approach on-pathway X-ray structures in both directions: the forward path crosses the 1YZ9 point as expected, and the reverse deviates along PC2 approaching the region populated by Mg\(^{2+}\)-bound structures. In fact, the transition from the pre-catalytic (1YYW) towards the catalytic state (2EZ6) is the next natural step along the RNaseIII cycle with Mg\(^{2+}\) present. This suggests a multi-step mechanism (Fig. 6a,b) agreeing with experimental models\(^{49}\) in which, as RNA binds (1YYO), the dsRBDs first separate along PC1 (crossing 1YZ9) and once they are wide-open, start to rotate along PC2 to reach 1YYW (90° rotation, forward). Then, as the bias (that is, Mg\(^{2+}\) in vivo) favours closing again (reverse), the dsRBDs naturally approach the catalytically competent region (180°) up to a point where they start to rotate back to resting position (1YYO). Thus, the eBDIMS trajectory suggests that the topologically accessible route to relax and close the protein naturally approaches the catalytically
Oryctolagus cuniculus sarco-endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). The SERCA pump is the best-studied P-type ATPase, which transport ions across cell membranes. There are >60 SERCA structures bound to ligands and ATP analogues, which cover nearly all the catalytic cycle. The cytosolic ‘headpiece’ domains, A (actuator), N (nucleotide binding) and P (phosphorylation) undergo translations and rotations as they bind and hydrolyse ATP. These motions are coupled to piston-like movements of transmembrane helices (TM) reshaping Ca\(^{2+}\) accessibility. The pump cycles between E1/E2 conformations: in the E1 state (E1-free), the Ca\(^{2+}\) high-affinity sites facing the cytoplasm are occupied (E1-2Ca\(^{2+}\)) favouring ATP binding (E1-2Ca\(^{2+}\)-ATP); then nucleotide hydrolysis triggers Ca\(^{2+}\) transport (E2-2Ca\(^{2+}\)) releasing ions into the lumen (E2-free). We focused on the transition from the open E1-2Ca\(^{2+}\) state (2C9M), with a splayed-headpiece, to the closed-headpiece E1-2Ca\(^{2+}\)-ATP structure (1T5S) (rMSD 14 Å) (Fig. 5a), locked onto an ATP-binding pocket. As with RNaseIII, the complexity and amplitude of the motions along the catalytic cycle yields similarly weighted components (Fig. 5b and Supplementary Table 2): PC1 (57% variance), which tracks most of the E1->E2 ion pumping, and PC2 (28% variance), which describes 95% of the A/P closure to bind ATP (Supplementary Table 6); both PCs correlate with heuristic variables (Supplementary Fig. 3d). Altogether, these motions separate structures into seven clusters (Fig. 5c, left), with E1-2Ca\(^{2+}\) showing great dispersion due to headpiece mobility.

For this transition, we were not aware of well-identified crystallographic intermediates. However, PCA neatly distributed structures into three groups along PC2: a cluster of E1-Mg\(^{2+}\) bound structures (4H1W, 3W5A and 3W5B), visited by the eBDIMS closing trajectory (Fig. 6d, Supplementary Table 5 and Supplementary Table 7). Although most methods track this transition (Fig. 6c, right), some become unstable in the intermediate region, where the lowest NMs from nucleotide-bound/intermediate states split onto two orthogonal directions (Supplementary Fig. 4c, Supplementary Table 7).

To further test the versatility of eBDIMS, we reconstructed the structure 4NAB, a catalytically incompetent mutant E309Q. This structure was not included in the ensemble since the
A-domain is missing, but the loops connecting to TM1-2 allowed eBDIMS to approximate its position (Supplementary Fig. 7a). Upon projection, 4NAB surprisingly appeared as a potential topological intermediate in the reverse path (Fig. 5c left, red dot). Although here intermediates are present in forward/reverse routes, the subtle asymmetry (0.15; Supplementary Fig. 5) and similar sampling of heuristic variables (Supplementary Fig. 7b,c), suggests identical opening/closing routes. Inspection of the MD FEL supports this view, and hints again that non-linear pathways delimit the edges of a single low-energy trough with X-ray intermediates on both sides (see below).

**Gloeobacter violaceus** ligand-gated ion channel (GLIC). Pentameric ligand-gated ion channels form a large family of membrane proteins with a central role in signal transduction, transmitting ligand binding through the opening of their ion-conducting pore. The proton-gated channel GLIC has been intensely studied as a model for eukaryotic counterparts. Crystals of closed GLIC were recently determined, which together with locally closed and open structures track the gating mechanism. Upon H⁺-binding, the extracellular domain undergoes a contraction (un-blooming) propagated to the intracellular region, triggering the tilting of pore-lining M2 helices in a cooperative iris-like motion that opens the gate; then...
the quaternary twist of the subunits locks the receptor in the conducting state.

The GLIC X-ray ensemble contained 46 near-intact pentamers (Supplementary Table 2). We selected the structures 4NPQ and 4HFI as representatives of the resting and H\(^+\)-bound conducting states, respectively (Fig. 7a). In this case the transition is subtle (rMSD = 2.66 Å) but requires cooperative motion of five subunits. Here the main PCs are similarly weighted (Fig. 7b); while PC1 (42% variance) tracks blooming-like motions of the extracellular domains, PC2 (30% variance) (Fig. 8a, left) describes quaternary twisting and pore gating (Fig. 8a, right), in accordance with the literature.\textsuperscript{31,35} (Supplementary Fig. 8a). Notably, structures are separated into functional clusters predominantly by PC2, which shows the strongest correlation with pore radius and contributes with up to 84% to the gating transition (Supplementary Table 6). The projections onto the PC1-2 subspace divide the ensemble into five well-defined clusters related to their functional status and crystallization conditions (Fig. 7c, left). Remarkably, the PCs split the locally closed as well as the open structures into two groups along PC1, which differ in their extracellular diameter (80 versus 70 Å) (Fig. 8b) suggesting two possible routes for gating: one ‘bloomed’ leading from 4NPQ to 4HFI, crossing a series locally closed structures (3TL\(^+\)) stabilized by Cys-bridges\textsuperscript{32} and a structure in equilibrium between locally closed/open (4NPPO\textsuperscript{31}); the other route, with ‘un-bloomed’ structures, leads from 4NPQ to the rightmost open cluster passing the locally closed 4LMJ and 4LMK. The fact that both groups distribute concentrically along a near diagonal axis suggests that they are functionally equivalent, and that the difference in compactness is due to their extracellular mobility in the crystal lattice. After examining crystallization conditions, we found that >90% of the bloomed structures were solved at a higher temperature (293K) than those un-bloomed at the right (277K). Only five outliers are found (Supplementary Fig. 8b): the open structures 4IRE and 4F8H, which appear in the high-temperature (bloomed) region but were solved at 277K, and the structures 4LMJ, 4LMK and 4LML, in the low-temperature (compact) region but solved at 298K. Interestingly, the first two correspond to GLIC bound to ketamine (4F8H) and loop C mutations (4IRE) that actually inhibit the channel. Although they are open in the TM region (pH 4), these pentamers appear more bloomed than other non-inhibited structures at 277K. In contrast, the un-bloomed 4LM\(^+\) locally closed and open structures are more compact than others at high-temperature. They harbour TM2-TM3 loop mutations designed to impair proton binding and gating: loss-of-function changes in the locally closed ones, and a double rescuer mutation in the open-like one.\textsuperscript{36} This suggests that extracellular blooming at a given temperature may be a subtle signature of the channel functional status, and certainly deserves further investigation.
The projection of the eBDIMS opening trajectory onto the PC1-2 subspace shows a smooth sampling of the motions encoded in the X-ray ensemble, sequentially visiting the locally closed intermediates (Fig. 7d); the ordering of locally closed structures by PCs perfectly agrees with their rMSD from the end points. Un-blooming precedes the quaternary twist decrease as previously suggested\(^{51,55}\) and in accordance with PCs (Supplementary Fig. 9a–e). Even in such a concerted multi-subunit transition, the motions are again encoded in low-frequency NMs (Supplementary Table 7). For GLIC, most methods are capable of sampling this small transition but nevertheless differ in their linearity when projected onto the PC1-2 subspace, with eBDIMS providing the broader sampling (Fig. 7c). There is once again only a subtle asymmetry of the reverse pathway (0.06; Supplementary Fig. 5): while the transition starting from resting GLIC proceeds through locally closed structures as the channel opens, the closing transition follows a path slightly left-shifted. Considering that bloomed locally closed and open structures are poorly separated by PC1, both pathways appear essentially equivalent, suggesting reversible forward/reverse routes for gating in which blooming and quaternary twist motions proceed in concerted fashion. As for SERCA and RBP, MD simulations suggest that both pathways explore the same low-energy passage connecting closed and open GLIC (see below).

Intermediates sampling in atomistic FELs. To explore the significance of pathway divergences on the PC-subspace, the sampled intermediates and how they relate to the lowest energy paths between end-states, we computed FELs from atomistic MD for three cases: (i) RBP hinge bending; (ii) SERCA headpiece closing and (iii) GLIC cooperative gating. These examples have been previously studied with MD\(^{57–59}\) and are known to reversibly transition between end-states in the absence of complex ligands, thus being suitable for comparison with eBDIMS. The atomistic methods to track these changes are also representative of common MD implementations (see Methods):

**Figure 6 | Comparison between the crystallographic and the eBDIMS pathways for RNaseIII and SERCA.** (a) Upper row: The complete X-ray structures for the RNaseIII end-states and the intermediate are shown bound to the dsRNA substrate; these conformations represent three sequential snapshots along the RNaseIII cycle. The starting structure is bound but catalytically inactive, and the following two show the progress to reorient the dsRNA onto the catalytic cleft (approximately in the z axis of symmetry). Lower row: as the two dsRNA-binding domains start to open the eBDIMS pathway shows how they spontaneously sample PC1 approaching the 1yz9 intermediate (shadowed); then, they leave it behind as rotation along PC2 progresses. (b) Superimposition between X-ray intermediate structure 1yz9 and the best overlapped eBDIMS frame. (c) Upper row: The complete X-ray structures for the SERCA open and closed end-states and one of the Mg\(^{2+}\)-bound visited intermediates (4h1w) are shown; the structure is approached within 4 Å (d) by the forward eBDIMS pathway tracking headpiece closing.
biasing along a heuristic reaction coordinate (RBP); standard MD from unbound structures (5'NTase); multi-run MD simulations (SERCA) and a single microsecond-trajectory (GLIC). For RBP, we collected transition pathways from end-states with steered MD and AWH28 using domain distance as reaction coordinate. In accordance with former studies57, trajectories from the closed structure (2DRI) rapidly converge on open and partially open conformations, while simulations from the open (1BA2) overlap with the former but never reach the fully closed state in the absence of ribose. The FEL for the opening transition (Fig. 9a) reveals that the lowest energy through connecting end-state basins actually comprises most of the area delimited by eBDIMS forward/reverse paths; interestingly, experimental intermediates are found along the edges of this region, indicating that they correspond to meta-stable states captured by crystallization but not to energy minima in solution. For SERCA, several MD studies have shown spontaneous Ca$^{2+}$-independent closing of the headpiece58,60 in the absence of bound nucleotide. Specifically, a recent computational study58 (also supported by FRET measurements) reported saltatory headpiece closure reaching long-lived states similar to the Mg$^{2+}$-bound structure (3W5B) here identified as an on-pathway intermediate. The E1-Mg$^{2+}$ configuration was suggested to approach an elusive E1-apo intermediate between E2-free and the E1-2Ca$^{2+}$ state, which would explain the accelerating effects of Mg$^{2+}$ on Ca$^{2+}$ binding61,62. According to the combined PCA/eBDIMS analysis, this configuration may as well represent a transient state in which Ca$^{2+}$ and ATP sites are pre-poised for efficient nucleotide binding. We compared the FEL sampled by MD58 from the open cluster (1SU4) which show spontaneous closing, with the eBDIMS pathways from/to 1T5S. Once again, the forward/reverse routes sample the boundaries of a wide low-energy area for

Figure 7 | Conformational transition of GLIC. (a) The two crystallographic structures representing the resting state (4npq) and the open conducting state (4hfi). (b) Dominant PCs of the GLIC X-ray ensemble (46 structures) versus 4npq. (c) Projection of the eBDIMS pathway onto the two major PCs of the ensemble (left); zoom highlighting the distribution of structures in the locally closed region (right). (d) Comparison between the forward pathways computed by eBDIMS, iENM, NOMAD-Ref, MinActionPath and Climber. Reverse pathways generated by eBDIMS, iMODS, NMSIM and Climber also shown.

a

b

PC1 (42%) - blooming

PC2 (30%) - twisting

c

d

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the closing transition (Fig. 9b), populated by crystallographic intermediates. Finally, we compared the GLIC eBDIMS pathways with a microsecond-long simulation from the open state that spontaneously closes at pH 7 (ref. 59), and a shorter 500 ns from the closed state at pH 4 that evolves to the open state. Inspection of the corresponding FELs (Fig. 9c,d) suggests again that both eBDIMS pathways delimit the same low-energy route connecting the open/closed basins and thus are equally significant. Notably, as in the former cases, crystallographic locally closed intermediates do not fall into energy minima but rather sample the boundaries of the low-energy region sampled in the closing transition.

Discussion
The aim of this work was to explore the possible reconstruction of protein transition pathways by prediction of intermediates from pairs of static structures. For that purpose, we developed a new sampling method, eBDIMS, and in parallel, a thorough PCA-based validation scheme to assess its biological relevance as well as to retrieve pathway information from structural ensembles. Although PCA has been used to extract motions sampled by MD, or to evaluate NMs from experimental ensembles, here it is applied to ensembles with enough sampled states to reconstruct the conformational landscape along meaningful CVs. We have shown that PCA of such ensembles automatically yields reaction coordinates that contain the one-dimensional system-defined parameters typically described for each protein. Moreover, the variance distribution informs on the dimensionality of the transitions: whether they are reducible to one (RBP or 5NTase) or a few coordinates (RNaseIII) or rather need several (GLIC) to be fully described. The complexity of the PCs emphasizes the risk of biasing trajectories using simple variables, and also suggests that a deeper study of ensemble-PCs can help to understand how complex motions are coupled in transitions. Furthermore, structure clustering by PCs can distinguish not only functional states, but also specific experimental conditions. For the GLIC ensemble, PCA clustering detected a previously unnoticed partition of the solved structures into two groups dependent on the two crystallization temperatures used for solving them (277 and 293K). The fact that the only outliers in both groups are either bound to molecules or harbour mutations to perturb channel activity suggests a possible link between intrinsic mobility of the extracellular piece and the channel status which is reflected in its compactness in the crystal lattice. Thus, the PCA method by itself can help to assign a functional status to new structures, understand how they relate to each other or even raise experimentally testable hypotheses.

More important, PC-clustering automatically provides the most probable sequence of structures along a transition avoiding uncertainties introduced when dealing with several heuristic variables in complex transitions such as GLIC. By extracting the common pattern of motion not for a pair but for all representative conformations for a protein, the PCs uncover the ensemble-encoded routes for conformational changes. Using the PC1-2 bidimensional space as reference for benchmarking, it is straightforward to evaluate how well sampling algorithms explore the conformational space. The projections onto the major PCs clearly distinguish feasible trajectories, characterized by a smooth and stable sampling of the experimental motions that spontaneously approaches intermediate states. On the basis of

Figure 8 | Correlations between structural variables for GLIC and the PC1-2 partitions reveal two possible temperature-dependent pathways for gating. (a) Variation of major structural parameters in the PC1-2 subspace (Pearson Correlation in brackets; further details in Supplementary Fig. 8a). (b) PC1 partitions the locally closed and open structures into two subsets according to the expansion of the extracellular domain; these two subsets strongly correlate with the crystallization temperature (Supplementary Fig. 8b). Bloomed structures are solved at 290 K while the more compact on the right are mainly solved at 270 K.
this stringent evaluation, we demonstrate that it is possible to reconstruct the structural landscape and predict possible intermediate structures using path-sampling algorithms. Notably, the CG eBDIMS trajectories greatly overlapped with the atomistic method Climber, based on pulling and energy minimization of the force-field ENCAD. The ability of a pure ENM topology-based simulation such as eBDIMS to predict intermediate states is a strong demonstration of the emerging paradigm that the large-scale functional dynamics of proteins is greatly encoded in their overall shape and does not depend on fine-grained sequence details.

While in some proteins (for example, RNaseIII) apparently there is one topologically accessible sequence of motions that allows for a transition, in others (for example, GLIC) several concerted motions proceed simultaneously until completion. Our results suggest that the ability to spontaneously sample a transition is pre-encoded in the overall shape and does not depend on fine-grained sequence details.

Figure 9 | Overlap of eBDIMS forward and reverse pathways with free energy landscapes obtained from atomistic simulations. (a) RBP opening with biased MD trajectories (1 μs); (b) SERCA headpiece spontaneous closing in multi-run MD simulations from the open conformation 1su4 (2 μs) reaching the intermediate state cluster; (c) GLIC spontaneous closing in a single long 1 μs-simulation at pH = 7 and (d) opening in a 500 ns simulation at pH = 4.6. Note that the slightly divergent pathways enclose most of the lowest energy passage between the end-states, with the approached crystallographic intermediates distributed at the boundaries.

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distorted pathways such as such motions are often not sampled experimentally. Nevertheless, most ENM-based algorithms perform extremely well providing fast approximations to the experimental paths in simple cases (see Supplementary Note 1).

Interestingly, both Climber and eBDIMS show nearly identical divergence in the forward/reverse pathways; however, considering PCs variance, this asymmetry is small and only for some examples hints to distinct sequences of motions as a protein progresses along its functional cycle. The latter is notable in the hysteresis-like cycle between RNaseIII RNA-bound structures (Fig. 4c), with nearby intermediates in both directions. In this extremely complex transition, eBDIMS and Climber suggests asynchronous motions (RBDs opening/rotation in the forward route, followed by closing/rotation in the reverse). Here pathway divergence, already evident from the PC1-2 distribution of experimental conformers, contains information about the sequence of movements in a multi-step complex landscape; unbiased MD from unbound forms (Supplementary Fig. 6c) also hints to differences in the preferred forward/reverse paths as suggested for some proteins. However, when asymmetry is low, comparison with the FELs from MD rather suggests the equivalence of forward/reverse routes: in the three cases examined, they appear to delimit not different linear paths, but rather an area in the conformational landscape that overlaps with the lowest energy passage connecting the end-state basins. Notably, the crystallographically trapped intermediates in all cases tend to distribute along the edges of these
regions, pointing that they correspond to meta-stable states but not to energy minima in solution. Apparently, transitions from open (enthalpy-driven) and closed (entropy-driven) forms provide two alternative but energetically feasible solutions for a path, indicating the usefulness of non-linear methods to explore lowest energy channels in the protein landscape.

With the rise of cryo-electron microscopy (cryo-EM) and time-resolved X-ray techniques capable of trapping structures in several conformations at once, the proteins solved in three states or more will no longer be anecdotic but rather standard.

In several conformations at once, the proteins solved in three states or more will no longer be anecdotic but rather standard. The first condition is Gaussian with zero mean, and second, its autocorrelation function has the form (see further details in refs 29,66):

\[ \langle \xi(t) \xi(t') \rangle = 2k_B T \theta(t-t') \]

Where \( k_B \) is the Boltzmann constant, \( T \) is the temperature of the stochastic bath (300K), and the \( \delta \)-Dirac functions ensure the independence of the components of the noise vector. Besides of representing the solvent, the friction and noise terms create a natural thermostat where random energy shots are balanced by the dissipative forces, keeping constant temperature and energy.

The stochastic equation of motion in (1) is integrated numerically with the Verlet algorithm, which gives for the velocities and positions after timestep \( \Delta t \)

\[ \frac{d^2 \mathbf{r}_i}{dt^2} = -\frac{\partial U}{\partial \mathbf{r}_i} - \kappa_i \mathbf{f}_i(t) \]

Where \( U \) is the potential energy function. The eBDIMS method can work with limited force-field descriptions of proteins, allowing for the efficient sampling of complex multidimensional data. The eBDIMS method can work with limited force-field descriptions of proteins, allowing for the efficient sampling of complex multidimensional data. The eBDIMS method can work with limited force-field descriptions of proteins, allowing for the efficient sampling of complex multidimensional data.

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are very robust even when considering a minimal number of structures (three) as discussed above (see Supplementary Table 2). Within this framework, any structure i is characterized by its projections onto the conformational space defined by the two major components, PC:\n
\[ \text{PC}_i = \{T_{i,a}, \cos(\text{PC}_k \land T_{i,a})\} \]

Where \( T_{i,a} \) is the difference between the coordinates of structure i and the chosen reference and \( \text{PC}_k \) is one of the major axes. Since we selected ensembles with structures sampling not only end states but also known intermediates, these major PCs actually describe the pathway for conformational change, and projection onto the new coordinates reveals the ordering of structures as a transition proceeds. The comparison between distances in rMDS and in the PC-I2 subspace to intermediates and target structures for the methods tested reveals subtle but relevant differences between the two measures (Supplementary Table 5). The Euclidean distance in terms of the PC coordinates provides an alternative metric, which weights the differences between any two given structures according to the relevant motions thus filtering out local fluctuations that contribute to the rMDS. Note the clear detection of intermediates in Figs 2–5 and 7 panel d, which is more pronounced than in the equivalent rMDS profiles.

Comparison to standard path-sampling algorithms. The eBDIMS trajectories are compared with other ENM-based methods of different complexity: NOMAD-Ref44, which uses ENMs to interpolate interresidue distances with the algorithm of Kim et al.43, MinActPath35, which solves analytically the Langevin equation for harmonic potentials on both sides of the transition and finds numerically the crossing points of the solutions; finally, the iENM46 is based on solving the saddle points of a double-well potential by linearly interpolating between the end-states ENM potential functions while iMODS40 interpolates in the dihedral angle space. We also compare with the non-linear atomic methods NMSIM34, which uses a complex three-steps procedure, and Climber, based on the molecular mechanics ENCAD potential33. All the methods were run with their default parameters (See Supplementary Table 4 and Supplementary Methods).

For the non-linear eBDIMS and Climber, trajectories were computed in both forward and backwards directions; and their asymmetry score (ranging from 0 to 1), evaluated as the eccentricity of the resulting ellipsoids in the PC-I2 subspace; the rMDS contour plots between the forward/reverse trajectories were also computed (see Supplementary Methods and Supplementary Fig. 5). Note that, in the PC-I2 subspace, a Cartesian interpolation such as that provided by the MoldMv server (Morph)39 projects as a straight line between the end-states regardless of the energetization of the structures (see summary of methods in Supplementary Table 4). To gain further insight into how motions are imprinted in the structures, we also created ensembles along the lowest frequency NM30 with a simple Monte Carlo routine.

MD simulations. Crystal structures of RBP (1BA2, 2DR1, 1URP), RNAseIII (1YYW, 1YYO, 1Y29), S-Ntase (1OID, 1O1B, 1HPU) and GLIC (4NPQ) were used for MD simulations. Each system was solvated with TIP3P waters, energy minimized and equilibrated, and production runs were carried out with no restraints under isothermal-isobaric (NPT) ensemble. Steered MD and AWH simulations of RBP were run under the same conditions as the production runs, with the same reaction coordinate (interdomain distance between center-of-masses). See detailed protocols in Supplementary Methods. Dr Seth Robia and Dr Marc Baaden generously provided trajectories for SERCA (1SU4) and GLIC (4HFI), respectively. The SERCA simulations were performed in explicit water under NPT conditions (\( T = 300\mathrm{K} \)) as described in detail in Smolin and Robia36. GLIC simulations were also performed in explicit water under NPT conditions as described in Nury et al.42. A list of all simulations and their overlap with eBDIMS is provided in Supplementary Table 8.

Calculation of heuristic system-defined structural variables. The heuristic system-defined variables for each protein were computed according to the specific literature using in-house tools combined with Visual Molecular Dynamics (VMD) scripts (see details in Supplementary Methods).

Data availability. The original FORTRAN code for eBDIMS and source data for all figures and tables is available upon request to the authors.

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