On the potential alternate binding change mechanism in a dimeric structure of Pyruvate Phosphate Dikinase

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The pyruvate phosphate dikinase (PPDK) reaction mechanism is characterized by a distinct spatial separation of reaction centers and large conformational changes involving an opening-closing motion of the nucleotide-binding domain (NBD) and a swiveling motion of the central domain (CD). However, why PPDK is active only in a dimeric form and to what extent an alternate binding change mechanism could underlie this fact has remained elusive. We performed unbiased molecular dynamics simulations, configurational free energy computations, and rigidity analysis to address this question. Our results support the hypothesis that PPDK dimerization influences the opening-closing motion of the NBDs, and that this influence is mediated via the CDs of both chains. Such an influence would be a prerequisite for an alternate binding change mechanism to occur. To the best of our knowledge, this is the first time that a possible explanation has been suggested as to why only dimeric PPDK is active.

Pyruvate phosphate dikinase (PPDK) is a key enzyme in the cellular energy metabolism that catalyzes the ATP- and phosphate (Pᵢ)-dependent conversion of pyruvate to phosphoenolpyruvate (PEP) in C₄ plants and the reverse, ATP-forming reaction in bacteria and protozoa. The reaction involves two independent partial reactions in plants1 and protozoa2, with a phosphoryl transfer mediated by the catalytic histidine H4563–5 (amino acid numbering according to the PPDK sequence of Flaveria trinervia):

\[
\text{Enzyme} + \text{ATP} + Pᵢ \rightleftharpoons \text{Enzyme—His—P + AMP + PP} \tag{1}
\]

\[
\text{Enzyme—His—P + Pyruvate} \rightleftharpoons \text{Enzyme + PEP} \tag{2}
\]

The temporal separation of the partial reactions is mirrored by a spatial separation of reaction centers6–7: PPDK consists of three domains, an N-terminal nucleotide-binding domain (NBD), the location of partial reaction (1), a C-terminal PEP/pyruvate-binding domain (PBD), the location of partial reaction (2), and a central domain (CD), the location of H456 (Fig. 1a). To date, 13 crystal structures of PPDK have been resolved8, for PPDKs of the bacterium Clostridium symbiosum7,9–12, the protozoan Trypanosoma brucei13, the C₄ plants Zea mays14 and Flaveria trinervia8, and the C₃ plant Flaveria pringlei8. Structural clustering of the PPDK structures reveals two principal movements (Supplementary Fig. S1): First, the NBD, which consists of three subdomains forming the ATP-grasp motif15, shows an opening-closing motion assumed to be associated with ATP binding13. Second, the CD is either located near the PBD, between the PBD and NBD, or near the NBD. Accordingly, and in line with the enzyme mechanism, the CD has been suggested to undergo a swiveling motion to shuttle the phosphoryl group between the two active sites, followed by a backward motion to initiate the next cycle of phosphoryl transfer7. The swiveling motion involves a rotation of ~110° of the CD, which transports H456 across a distance of ~45 Å. This makes the swiveling motion of PPDK one of the largest single-domain movements observed in proteins yet.

Recently, we predicted by unrestrained molecular dynamics (MD) simulations and 1D and 2D configurational free energy (potential of mean force (PMF)) calculations on monomeric PPDK that the swiveling motion proceeds...
via a conformational intermediate where the CD is located roughly in between the PBD and NBD. A crystal structure of PPDK from *Flaveria trinervia* confirmed this, until then, unknown intermediate (PDB ID 5JVJ). The molecular simulations and crystallographic data furthermore indicated that PPDK might employ a Brownian ratchet mechanism biasing thermal fluctuations in order to generate a net directional CD motion. Finally, our calculations revealed an intramolecular coupling between the CD motion and the opening-closing motion of the NBD, which was corroborated by the comparative analysis of available PPDK crystal structures. While the biologically active form of PPDK has been considered a dimer in bacteria, and may be a tetramer in several plants, the 5JVJ structure exposed for the first time the dimeric form of PPDK in the asymmetric unit, with the dimer interface formed by the two PBDs. Notably, the two monomers differ in the conformational state of the NBDs, with that of chain A being in an open and that of chain B in a closed conformation. The NBD of monomer B exhibits additional electron density that might reflect a bound adenine nucleotide. The distinct conformational states of the NBD of 5JVJ have led to the hypothesis that PPDK employs an alternate binding change mechanism (also termed reciprocating mechanism stressing its processivity) similar to ATP synthase or bacterial ATP-dependent DNA helicases. However, no further detailed insights if and how such an alternate mechanism operates in PPDK are currently available.
binding change mechanism occurs in a dimeric PPDK structure have yet been presented. Furthermore, it has remained elusive if such a mechanism underlies the activation of PPDK in the dimeric form. Here, we investigate the structural dynamics of the PPDK dimer from F. trinervia by unrestrained all-atom MD simulations of an aggregate simulation time of 3.6 μs (Table 1), the energetics of the opening-closing motion of one NBD as a function of the open/closed state of the other NBD as well as the conformational state of the CD in the PPDK dimer by PMF calculations of in total 2.6 μs sampling time (Supplementary Table S1), and the influence of dimerization on the structural stability by rigidity theory-based Constraint Network Analysis (CNA). Our computations support the hypothesis that PPDK dimerization does influence the opening-closing motion of the NBDs, and that this influence is mediated via the CDs of both chains.

### Results and Discussion

#### Native interface of the PPDK dimer.

Initially, we investigated the interface found between the PBD domains in 5JVJ as to its role for forming the dimeric biological unit of F. trinervia PPDK. Using the PDBePISA tool, the analysis revealed that this interface is the only one in this crystal structure suggested to be functionally relevant (complexation significance score (CSS): 0.541; CSS of all other interfaces: zero), with an interface area of 2077 Å², > 40 potential hydrogen bonds and salt bridges and > 65 potential hydrophobic interactions formed across the interface, and a ΔG_p-value of 0.370 (Supplementary Table S2). Furthermore, mutations reported to reduce PPDK’s cold-dependent dissociation and, therefore, inactivation are located in the vicinity of the dimer interface (Supplementary Fig. S2a), and surface residues forming the dimer interface are conserved (between 25% and 100%, Supplementary Fig. S2b and Supplementary Table S3). The only other two regions with similarly high conservations of surface residues are in the vicinities of the functionally relevant active sites of the NBD and the PBD, where the CD docks during the swiveling motion (Supplementary Fig. S2b). The high degree of sequence conservation thus correlates with the functional importance of these regions. Together, these results imply that the interface found between the PBD domains plays an essential role in complex formation rather than it being a result of crystal packing only.

#### Structural dynamics of dimeric PPDK.

Next, we generated all-atom structures of PPDK dimers from F. trinervia with the two NBDs in unbound closed/closed, open/closed, or open/open conformations, as well as in ATP-bound closed/closed conformations. The dimers served as starting structures for three independent MD simulations of 300 ns each (Table 1), respectively, in total, resulting in twelve MD simulations (Fig. 1b). The setup in terms of force field and simulation parameters was identical to the one used successfully in ref. 8 on monomeric PPDK, applying, in addition, force field parameters for ATP and Mg²⁺ (see Materials and Methods for details). The MD simulations revealed a pronounced tendency for unbound, closed NBD domains to open (six out of nine cases) with an opening usually occurring during the first 150 ns of a trajectory. In contrast, open NBD conformations remained open during the simulation time (nine out of nine cases), and ATP-bound closed conformations remained closed (six out of six cases), although it cannot be excluded that this finding is due to too short simulation times, particularly in the last case. Still, with respect to the question of an alternate binding change mechanism, ten out of twelve MD simulations do not indicate a preference for one NBD of the PPDK dimer to be in a conformation different from the other, given identical binding states of both domains. Note that the CDs stayed at their starting positions facing the PBD in all MD simulations, in contrast to the observation from our study on monomeric PPDK. The latter observation might result from MD simulation times that are at least two-fold longer than those here.

Furthermore, a principle component analysis (PCA) in Cartesian space was performed on coordinates derived from all twelve MD simulations. 66% of the variance in the motions of the PPDK dimers can be characterized by the first two principal components (PC), with the 1st and 2nd PC explaining ~34%, and ~32%, respectively (Supplementary Fig. S3a). According to atomic displacements along PC directions (Fig. 2a), both the 1st and 2nd PC primarily reflect the opening-closing motions of both NBDs, which are predominantly executed by the first and second subdomains (NBD1 and NBD2), as indicated by (sub)domain-wise collectivity indices (Supplementary Fig. S3b). Interestingly, while the 1st PC describes an antisymmetric motion of both NBDs (i.e., one opens, the other closes), the 2nd describes a symmetric one (i.e., both open, or close, simultaneously) (Fig. 2a,b,c). Considering the almost equal proportion of variance both PCs characterize, this finding implies that the opening-closing motion of one NBD is not directly dependent on the opening-closing motion of the other NBD.

Moreover, cross-correlation analysis of α(atom fluctuations, computed to identify a possible coupling between the opening-closing motions, reveals, first, positively correlated motions of the PPDK domains (NBDs, CDs, and PBDs) themselves, and, second, in part positively and in part weakly negatively correlated motions between the PBDs (Fig. 2b); the positively correlated motions occur in those regions of the PBDs that form the dimer interface (Supplementary Fig. S4b). Third, motions of the NBD of one chain are weakly negatively correlated to those of

| Conformation chain A | Conformation chain B | Repetitions | Simulation length [ns] |
|----------------------|----------------------|-------------|------------------------|
| NBD closed           | NBD closed           | 3           | 300                    |
| NBD open             | NBD closed           | 3           | 300                    |
| NBD open             | NBD open             | 3           | 300                    |
| NBD closed, ATP bound| NBD closed, ATP bound| 3           | 300                    |

Table 1. MD simulations of the PPDK dimer. [a] In ns.
the PBD of the other chain, which might reflect the close distances between these domains (Fig. 1a). However, motions between the NBD of one chain and the PBD of the same chain are largely uncorrelated, as also found for monomeric PPDK. Motions between both CDs are largely uncorrelated as well, as may have been expected from the CDs moving little with respect to their starting position (see above). Finally, motions between the two NBDs...
are also uncorrelated (mean correlation coefficient of 0.1), implying again that the opening-closing motion of one NBD is not directly dependent on the opening-closing motion of the other NBD.

**Energetics of the opening-closing motion.** To complement the unrestrained MD simulations, we computed the PMF of the opening-closing motion of the NBD of chain A, with chain B having the CD facing the PBD with the NBD in the open, unbound conformation (a, black circle), closed, unbound conformation (b, blue triangle pointing upwards), and closed, ATP-bound conformation (c, green square), as well as the CD facing the NBD with the NBD in the closed, unbound conformation (d, orange triangle pointing downwards) and closed, ATP-bound conformation (e, red diamond). The distance $\text{NBD}_{1}$-$\text{NBD}_{3}$ between Ser215C$_{\alpha}$ – Glu272 C$_{\alpha}$ was used as a reaction coordinate (depicted as black arrows in panel a). The standard deviation for all data points is $< 0.02$ kcal mol$^{-1}$ computed by a bootstrapping procedure (see Methods section).

![Figure 3.](image)

**Figure 3.** PMF of the opening-closing motion of chain A for different states of chain B. (a) Schematic representation of simulated systems and (b) PMF of the opening-closing motion of the NBD of chain A, with chain B having the CD facing the PBD with the NBD in the open, unbound conformation (a, black circle), closed, unbound conformation (b, blue triangle pointing upwards), and closed, ATP-bound conformation (c, green square), as well as the CD facing the NBD with the NBD in the open, unbound conformation (d, orange triangle pointing downwards) and closed, ATP-bound conformation (e, red diamond). The distance $\text{NBD}_{1}$-$\text{NBD}_{3}$ between Ser215C$_{\alpha}$ – Glu272 C$_{\alpha}$ was used as a reaction coordinate (depicted as black arrows in panel a). The standard deviation for all data points is $< 0.02$ kcal mol$^{-1}$ computed by a bootstrapping procedure (see Methods section).
(Fig. 3), corroborating the above implications (Figs 1 and 2) that the opening-closing motion of one NBD does not directly depend on the conformational and binding state of the NBD of the other chain.

Changes in structural stability due to PPDK dimer formation. As an independent way to analyze mechanical coupling within the PPDK dimer structure, we performed CNA on coordinates of chain A, chain B, or the dimer, which were all extracted from one MD simulation of the PPDK dimer with a conformation as observed in the X-ray structure (PDB ID 5JVJ) (see Materials and Methods for details). In CNA, the molecular system is represented as a constraint network, which is analyzed using rigidity theory, revealing rigid clusters and flexible links in between. By performing a constraint dilution simulation, a stability map is obtained that reports on the hierarchy of structural stability of the molecular system. It does so in terms of the energy along a constraint dilution trajectory at which a rigid contact between a pair of amino acids vanishes; a rigid contact is present as long as the two residues belong to the same rigid cluster (Supplementary Fig. S6). The difference stability map calculated as $r_{ij}$ (Dimer) – $r_{ij}$ (Monomer, chain A) – $r_{ij}$ (Monomer, chain B) then reports on the influence on structural stability due to dimerization. Here, only additional interactions across the interface affect the structural stability but not conformational changes, as the conformations of the dimer and the two monomer chains were extracted from one MD trajectory. Note that this analysis allows revealing long-range aspects to rigidity percolation, that is, whether a region is flexible or rigid may depend on structural details that are faraway.

The intermolecular part of the difference stability map was projected onto the PPDK dimer structure in terms of struts connecting respective residue pairs; the color of the struts indicates to what extent a rigid contact was stabilized due to formation of the PPDK dimer from the monomers (Fig. 4 and Supplementary Fig. S7). Dimerization does not only increase structural stability of both PBDs, as may have been expected because of additional interactions across the interface, but also that of the CD of one chain (Fig. 4f and Supplementary Fig. S7f), or the NBD of one chain (Fig. 4a,e and f and Supplementary Fig. S7c,g), due to the presence of the PBD of the other chain, respectively, as may have been inferred from the close distances between the respective domains. Effects on structural stability due to dimerization are also observed for residue pairs on the NBD of one chain and the CD of the other chain (Fig. 4b,d and f and Supplementary Fig. S7f,h). This finding clearly reflects the long-range aspect to rigidity percolation, as the distance between both domains is

![Figure 4](image-url)
at least 50 Å but additional constraints due to dimerization are only placed to model (short-range) non-covalent interactions (i.e., hydrogen bonds, salt bridges, and hydrophobic interactions between the monomers). Notably, however, no change in structural stability due to dimerization is observed for pairs of residues located on either NBD (Fig. 4c,f and Supplementary Fig. 7i).

**Coupling of the opening-closing motions mediated by the CDs.** Although we did not find evidence for a direct coupling of the opening-closing motion or the structural stability of the two NBDs, the fact that PPDK dissociates in a cold-dependent manner and then becomes inactive leads to the question as to why only the PPDK dimer is active. Previously, we showed for monomeric PPDK that the CD motion and the opening-closing motion of the NBD is coupled. Above, we showed by CNA that the structural stability of the CD and the NBD of one monomer is influenced by the presence of another monomer (Fig. 4a,b,d,e and f). Taken together, it is tempting to hypothesize that PPDK dimerization influences the opening-closing motions of the NBDs, and that this influence is mediated via the CDs of both chains (Supplementary Fig. S9). Initial support for this hypothesis is found by comparing the PMFs of the opening-closing motion of the NBD, when the CD is near the PBD, in the monomeric state (ΔG_{open → closed} ≈ 20 kcal mol⁻¹; see Fig. 6 in ref. 8) and in the dimeric state (with the CD of the other chain facing the PBD = state a), ΔG_{open → closed} ≈ 16 kcal mol⁻¹; Fig. 3). The comparison indicates that the closing of the NBD is less energetically unfavorable in the dimeric state, which could explain why the PPDK dimer is the active form. Further support for this hypothesis arises from additional PMF computations of the opening-closing motion of the NBD (chain A) of dimeric PPDK, in which the CD of the same chain faces the PBD but that of chain B faces the NBD (states d and e). The PMF of state d) reveals a lower free energy for the closing of the NBD of chain A (by ~6 kcal mol⁻¹ compared to states a) - c) at distance_{CD1-NBD1} ~ 26 Å; Fig. 3). The ATP-bound conformation of chain B (state e), which is expected to stabilize the conformation of the NBD and CD of chain B, fosters this effect in that the free energy for the closing of the NBD chain A is now lower by ~10 kcal mol⁻¹ (at distance_{CD3-NBD3} ~ 26 Å; Fig. 3) compared to states a) - c). Taken together with our previous results, these findings suggest that the conformational states of the CDs of both chains in the PPDK dimer affect the energetics of the opening-closing motions of the two NBDs. However, a thorough validation of this hypothesis would require PMF computations considering both distance_{NBD1-NBD3} as well as two reaction coordinates characterizing the swirling motion of each CD simultaneously, resulting in a 4D-PMF. Given the computational burden we faced when performing 2D-PMF calculations on monomeric PPDK, such calculations are beyond the scope of the present work, although alternative sampling methods may alleviate some of this burden.

Finally, our results lead to the question as to why the two NBDs in the asymmetric unit have been found with distinct conformational states in the crystal structure PDB ID 5JVJ although both CD do face the respective PBDs. A qualitative analysis of the crystal packing environments of the NBDs suggests that the crystal packing contributes favorably to, if not fosters, the occurrence of the conformationally distinct states of the NBDs (see section Supplementary Results, Supplementary Table S4, and Supplementary Fig. S8).

**Concluding remarks.** Results from unbiased MD simulations, cross-correlation analysis, PCA, PMF computations, and CNA did not provide evidence for a direct coupling of the opening-closing motion or the structural stability of the two NBDs, the fact that PPDK dimerization does influence the opening-closing motion of the NBDs, and that this influence is mediated via the CDs of both chains. Such an influence would be a prerequisite for an alternate binding change mechanism to occur (Supplementary Fig. S9) and could result in an overall mechanism of dimeric PPDK as displayed in Supplementary Figure S10. To the best of our knowledge, this is the first time that a possible explanation has been suggested as to why only dimeric PPDK is active. The suggestion would add another example to the rare occurrence of asymmetric organization of protein structures, and, as in other cases, the asymmetry would serve a special role, here enzyme activation. Besides further comprehensive PMF computations, it should be interesting to investigate the structural dynamics and function of dimeric PPDK by single molecule spectroscopy, applying, e.g., Förster resonance energy transfer measurements, in order to map the timescales of exchange and the pathways between conformational states.

**Methods**

**Generation of all-atom dimeric PPDK structures.** The crystal structure of the PPDK dimer with the NBD of chain A in the open and the NBD of chain B in the closed conformation now available from the Protein Data Bank (PDB ID 5JVJ) was used in all analysis. As 86 residues (9 in chain A and 77 in chain B) out of in total 1748 residues have not been resolved in this structure, a model containing all atoms was generated using the program MODELLER. For this, a sequence alignment was prepared considering the parts resolved in the crystal structure, and the missing parts by the template structure PDB ID 1VBG (NBD open) for chain A and the template structure PDB ID 5JVL (NBD closed) for chain B. Additional models of the PPDK dimer with both NBDs closed, and with both NBDs open, were generated by aligning respective monomers of the 5JVJ structure to the residues of the dimer interface (see Supplementary Table S3).

**Molecular dynamics simulations of dimeric PPDK.** MD simulations were performed using established protocols as described previously. In detail, co-crystallized water and ligands were removed from the PPDK dimer structures. Hydrogen atoms were added using REDUCE, flipping side chains of Asn, Glu, and His and assigning ionization states of Asp, Glu, and His when appropriate. These model systems were placed in a truncated octahedral box of TIP3P water leaving a distance of at least 11 Å between the solute and the border of the box. Counter ions were added to neutralize the systems. All MD simulations were performed with the ff99SB force field using the Amber suite of programs and the GPU version of PMEMD. Force field parameters for ATP were obtained from Meagher et al., force field parameters for Mg²⁺ were taken from Åqvist. Bonds
containing hydrogen atoms were constrained using the SHAKE algorithm, and long range interactions were treated by the particle mesh Ewald (PME) method. A time step of 2 fs was used. The system was equilibrated by, first, applying harmonic restraints to solute atom positions with force constants of at least 5 kcal mol$^{-1}$ Å$^{-2}$ for 100 steps of steepest descent and 400 steps of conjugate gradient minimization. Second, the temperature of the system was raised from 100 K to 300 K in 50 ps of NVT-MD simulations. Third, 150 ps of NPT-MD simulations were performed to adjust the system density. Finally, the force constants of harmonic restraints were gradually reduced to zero during 250 ps of NVT-MD simulations. Production NVT-MD simulations were carried out at 300 K, using the Berendsen thermostat and a coupling constant of 0.5 ps. Three independent replicates of MD simulations were performed for each system by spawning production runs after the thermalization at temperatures of 299.9 K, 300.0 K, and 300.1 K respectively. The first 2 ns of each trajectory were omitted from subsequent analyses. All MD simulations are listed in Table 1.

**Principle component analysis.** The principle component analysis in Cartesian space was performed on snapshots of MD simulations of the PPDK dimer with an aggregate length of 3.6 μs using CPTRAJ in a similar manner as described in ref. 8. In detail, an RMS-fit using the 15% least fluctuating residues was performed prior to the analysis to remove global translational and rotational motion. The coordinate covariance matrix was calculated for all C$_\alpha$ atoms. The symmetric matrix was diagonalized by an orthogonal coordinate transformation, yielding the eigenvalues and eigenvectors (principle components). An eigenvalue corresponds to the mean square eigenvector coordinate fluctuation (the variance) and, hence, describes how much a principal component contributes to the total coordinate fluctuations.

To analyze the locality or collectivity of motions for the domains of PPDK, the collective index $\kappa$ described in refs. 50 and 51 was calculated (equation 3)

$$\kappa = \frac{1}{N} \exp \left\{ -\sum_{i=1}^{N} \frac{\Delta r_i^2}{\kappa} \right\}$$

with $N$ being the number of atoms in the (sub)domain, and $\Delta r_i$ being the relative displacement of the principle component. All values of $\Delta r_i$ were scaled consistently such that $\sum_{i=1}^{N} \Delta r_i^2 = 1$. A value of $\kappa = 1$ indicates a mode of maximal collectivity, that is, all $\Delta r_i$ are identical. Conversely, if only one atom is affected by the mode, $\kappa$ reaches the minimal value of 1/N.

**Generation of transition paths.** For the potential of mean force computations, plausible pathways of the opening-closing motion have been generated in a similar way as described in ref. 8 using targeted normal mode-based geometric simulations by the NMSim approach. In detail, NMSim is a three-step protocol for multiscale modeling of protein conformational changes that incorporates information about preferential directions of protein motions into a geometric simulation algorithm. In the first step, the protein structure is coarse-grained to the analysis to remove global translational and rotational motion. The coordinate covariance matrix was calculated for all C$_\alpha$ atoms. In the second step, low-frequency normal modes are computed by rigid cluster normal mode analysis (RCNMA) with a 10 Å distance cutoff for considering interactions between C$_\alpha$ atoms. In the third step, a linear combination of the first 50 normal modes was used to bias backbone motions along the low-frequency normal modes, while the side chain motions were biased towards favored rotamer states, generating 100 conformational states in 100 simulation cycles with a step size of 0.5 Å and side chain distortion of 0.3. Targeted NMSim computations were performed for five states, which vary in the conformation of chain B. For chain A, the open state and the closed state of the NBD was used as start and end conformations for all systems, respectively; the CD of chain A was facing the PBD in all simulations. The start and end conformations of chain B were in state a): NBD in an unbound and open conformation with the CD facing the PBD; in state b): NBD in an unbound and closed conformation with the CD facing the PBD; in state c): NBD in an ATP-bound and closed conformation with the CD facing the PBD; in state d): NBD in an unbound and closed conformation with the CD facing the NBD; in state e): NBD in an ATP-bound and closed conformation with the CD facing the NBD (see Fig. 3a). State c) resembles what has been found in PDB ID 5JV.

**Potential of mean force computations.** Free energy profiles of the opening-closing motion of the NBD of chain A were computed along the NMSim-generated transition paths with further reference points added by using the conformation after 3 ns of umbrella sampling as a starting point for the next interval followed by the Weighted Histogram Analysis Method (WHAM). All umbrella sampling simulations with ATP bound to the NBD of chain B also contained Mg$^{2+}$ in the NBD of chain B. Possible interference between the NBDs was investigated by performing multiple umbrella sampling/PMF calculations of the opening-closing motion of chain A for systems with chain B in states a) - e) (see above). The opening-closing motion of the NBD was analyzed along the reaction coordinate $\text{distance}_{\text{NBD1-NBD3}}$ measured between S215C$_\text{NBD1}$ - E272G$_\text{NBD3}$ which has been shown to represent the opening-closing motion of the NBD very well (see Supplementary Fig. 4e in ref. 8). Umbrella sampling MD simulations were performed for $\text{distance}_{\text{NBD1-NBD3}}$ between 26 Å and 42 Å in intervals of 1 Å, applying harmonic potentials with a force constant of 1 kcal mol$^{-1}$ Å$^{-2}$ to tether the conformations to the respective reference point. This resulted in 17 umbrella sampling simulations, each 20 ns long, excluding the first 1 ns from the WHAM analysis. Approximately Gaussian-shaped frequency distributions were obtained for each reference point along the reaction coordinate, with all such distributions well overlapping (Supplementary Fig. S5). The latter is a prerequisite for the successful application of WHAM to extract a PMF from these distributions. The Monte Carlo
bothe analyses implemented in WHAM using 200 resampling trials was applied to establish the errors at the reference points. The umbrella sampling simulations are summarized in Supplementary Table S1.

Constraint Network Analysis. Constraint Network Analysis (CNA) is a graph theory-based rigidity analysis that links biomolecular structure, (thermo-)stability, and function. In the CNA approach, a protein is represented as a constraint network with bodies (representing atoms) connected by sets of bars (constraints, representing covalent and noncovalent interactions). A rigid cluster decomposition of the constraint network then identifies rigid parts that are connected by flexible links. By gradually removing noncovalent constraints from an initial network representation of a biomolecule, a succession of network states is generated that yields a ‘constraint dilution trajectory’. Analyzing such a trajectory by rigidity analysis reveals a hierarchy of rigidity that reflects the modular structure of biomolecules in terms of secondary, tertiary, and quaternary structure.

We applied CNA to investigate the effect of the dimerization on the structural stability of PPDK using three sets of coordinates extracted from one MD simulation of the PPDK dimer: coordinates of only chain A, of only chain B, and of both chains. For this, the first 200 ns of the MD simulation started with the PPDK dimer in the open/closed conformation of the NBDs and equilibrated at 300.0 K was used, where the conformations of the NBDs remain particularly stable (MD simulation started from open/closed and unbound conformation no. 2 shown in Fig. 1b; mean distance NBD1-NBD3 of 38.7 Å (SEM ~1.0 Å) and 31.3 Å (SEM ~1.0 Å) for chain A and B, respectively). With CNA, constraint dilution simulations were performed on ensembles of 10,000 structures each, of which water molecules and counter ions had been stripped off. A stepwise decrease of the energy cutoff $E_{\text{cut}}$ for including hydrogen bonds from $-0.1 \text{ kcal mol}^{-1}$ to $-6 \text{ kcal mol}^{-1}$ in steps of $0.1 \text{ kcal mol}^{-1}$ was performed.

Analysis of CNA results. Stability maps $r_{ij}$ were introduced in ref. 56 to characterize the local rigidity of a biomolecule. A stability map depicted rigid contacts ($r_{ij}$) for each residue pair $(i, j)$, represented by the Cα atom, respectively. A rigid contact exists if the two residues belong to the same rigid cluster. Notably, this stability information is not only a qualitative but also quantitative measurement. By performing constraint dilution simulations, each rigid contact is associated with an energy $E_{\text{cut}}$ at which this rigid contact is lost.

Data availability. All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author Contributions

D.C. performed research. D.C. and H.G. designed research, analyzed data, and wrote the paper.

Additional Information

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