

Folding and Unfolding of γ TIM Monomers and Dimers

Brijesh Patel and John M. Finke

Department of Chemistry, Oakland University, Rochester, Michigan

ABSTRACT Kinetic simulations of the folding and unfolding of triosephosphate isomerase (TIM) from yeast were conducted using a single monomer γ TIM polypeptide chain that folds as a monomer and two γ TIM chains that fold to the native dimer structure. The basic protein model used was a minimalist Gō model using the native structure to determine attractive energies in the protein chain. For each simulation type—monomer unfolding, monomer refolding, dimer unfolding, and dimer refolding—thirty simulations were conducted, successfully capturing each reaction in full. Analysis of the simulations demonstrates four main conclusions. First, all four simulation types have a similar “folding order”, i.e., they have similar structures in intermediate stages of folding between the unfolded and folded state. Second, despite this similarity, different intermediate stages are more or less populated in the four different simulations, with 1), no intermediates populated in monomer unfolding; 2), two intermediates populated with β_2 – β_4 and β_1 – β_5 regions folded in monomer refolding; 3), two intermediates populated with β_2 – β_3 and β_2 – β_4 regions folded in dimer unfolding; and 4), two intermediates populated with β_1 – β_5 and β_1 – $\beta_5 + \beta_6 + \beta_7 + \beta_8$ regions folded in dimer refolding. Third, simulations demonstrate that dimer binding and unbinding can occur early in the folding process before complete monomer-chain folding. Fourth, excellent agreement is found between the simulations and MPAX (misincorporation proton alkyl exchange) experiments. In total, this agreement demonstrates that the computational Gō model is accurate for γ TIM and that the energy landscape of γ TIM appears funneled to the native state.

INTRODUCTION

Many recent advances in understanding protein folding have been provided by minimalist simulations (1–18). One fundamental idea guiding many simulations is that natural selection has evolved each protein such that amino acids found in close proximity in the native structure of a protein are attractive (16,19–25). The attractive energies between distant residue pairs in the native protein structure of a protein have been selected against so that they are zero (2). This basic model has been called the Gō model (26).

The design and implementation of Gō-model simulations is relatively straightforward (16,21,25). However, what is intriguing about these simple models is that they reproduce the structures and stability of transition states and transient intermediate states in the folding of a number of proteins, as confirmed with various experiments (16,17,19,21–23). Having established this agreement with these small monomeric proteins, theoretical biophysicists are currently pursuing extensions of the Gō model to other, more complicated biomolecular interactions. The problems under study include large protein monomers, protein binding and assembly, multimeric protein complexes, protein-DNA complexes, and chaperonins (16–18,27).

This study investigates a relatively unexplored area of theoretical protein folding—the folding and binding of large (>200 amino acid) proteins into the dimeric native state. Thus far, many small protein folding simulations have been

published (19,21–23). In addition, a few theoretical simulation studies of protein binding have also demonstrated the success and future promise of the Gō model (17). Furthermore, a recent theoretical study of the α -subunit of tryptophan synthase (α TS), a large triosephosphate isomerase (TIM) barrel monomer, showed excellent agreement with a number of equilibrium and kinetic experiments (16). As a test case to investigate large protein dimers, the folding and binding of TIM from yeast (γ TIM) is explored in this study.

TIM catalyzes the fifth step in the glycolysis pathway and converts dihydroxyacetone phosphate (DHAP) into 3-glyceraldehyde phosphate (28). Since glycolysis is the most fundamental pathway through which organisms derive ATP from food sources, TIM proteins are found in nearly all organisms on earth (28). Furthermore, the structure of TIM, the TIM barrel, has proven to be useful in catalyzing many other metabolic biochemical reactions as well (28). Consequently, TIM has many paralogs within the same organism and across the genomes of most living species. This structural promiscuity has resulted in the notable finding that 10% of all protein structures in the Protein Data Bank (PDB) are TIM barrels. Therefore, an understanding of the fundamental physical forces that determine the folding and oligomer assembly pathways of TIM barrels can be applied to many known TIM barrel systems. Demonstration of the predictive success of a biophysical model to a test set of TIM barrels will provide the backbone for an exhaustive theoretical analysis of TIM barrel folding and binding across many genomes. This study investigates whether the Gō model is capable of predicting the folding and binding of γ TIM, a TIM-barrel protein, using experimental data on the number

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Address reprint requests to John M. Finke, Dept. of Chemistry, Oakland University, Rochester, MI 48309-4477. Tel.: 248-370-3088; E-mail: finke@oakland.edu.

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of folding intermediates in the folding pathway and also the structures of these intermediates.

After the first TIM-barrel x-ray crystallographic structures were obtained, the stability and folding pathways of TIM barrel proteins became a subject of scientific interest. The most simple TIM-barrel structure consists of eight repeating $\beta\alpha$ units, with the β -strands linked through hydrogen bonds like flat fence posts in a circular arrangement, such that the N-terminal and C-terminal β -strand bend around to “close the gate” by hydrogen-bonding with one another (28). The eight α -helices lie on the outside of the barrel shape made by the eight β -strands. At first glance, it might appear that all eight $\beta\alpha$ units would be required for the protein to be stable, since all eight strands are required to complete the TIM barrel’s circular arrangement and connect the - and C-terminal strands. Removal of any of the eight $\beta\alpha$ units would leave the TIM-barrel protein too short to bend around and make the N-C connection. Without this stabilizing connection, one might think that truncation mutants of TIM barrels would lack the stability to remain folded. However, truncation mutants of TIM-barrels can exist as stable structures (29–31). Thus, the stabilizing interactions may exist unequally throughout the protein chain. During the folding of TIM barrels, such interactions may give rise to unexpected folding pathways and intermediates.

Folding experiments of monomeric TIM barrels have been conducted on a number of TIM-barrel proteins. These include folding studies of yeast TIM (γ TIM), rabbit muscle TIM (rTIM), and *Trypanosoma brucei* TIM (tbTIM), the α -subunit of *Escherichia coli* tryptophan synthase (α TS), *Sulfolobus solfataricus* indole-3-glycerol phosphate synthase (sIGPS), *E. coli* IGPS (eIGPS), *E. coli* phosphoribosylanthranilate isomerase (PRAI), and rabbit muscle aldolase (29,31–48). These studies have revealed some similarities between the TIM-barrel folding pathways but also many differences.

A similarity between all TIM-barrel proteins studied is that their folding pathways do not follow a simple two-state mechanism and appear to always involve kinetic folding intermediates. Kinetic folding studies of all TIM barrels studied demonstrate multiphasic folding pathways that are found to be consistent with folding intermediates (37–39, 41,42,46,47). In addition, thermodynamically stable intermediates are also observed in equilibrium unfolding experiments of most TIM barrels (33–35,38,39,41,45,48,49). The possible exception of a TIM barrel with no thermodynamic intermediate is rTIM (42).

However, from a structural standpoint, the folding pathways of TIM barrels appear to be as different from each other as any one is from any group of unrelated proteins. Although the TIM barrels fold through intermediates, these intermediates appear to be quite different in structure. The equilibrium intermediates of α TS indicate that folding initiates at the N-terminus with an early folding intermediate, I_2 , within the region α_0 – α_4 (29,44), followed by an intermediate I_1

comprising regions α_0 – $\beta_6 + \beta_7$ (29,43,50). Kinetic folding studies of α TS also indicate early N-terminal structure in regions α_0 – $\beta_6 + \beta_7$ (37,50). A similar “N-terminus first” equilibrium folding pathway was found in γ TIM, the subject of this study. With γ TIM, folding initiates with an early intermediate, I_2 , comprising the region β_2 – β_4 followed by an intermediate I_1 comprising the region α_1 – β_6 (34).

Although this folding pathway might be found in other TIM barrels, it certainly does not apply to all TIM-barrel proteins. For example, for rTIM, no intermediates are observed in equilibrium unfolding, and in kinetic folding studies, the C-terminal region β_5 – α_8 appears to fold first (42). In addition, a dialysis refolding experiment indicates that the folding pathway of rabbit-muscle aldolase populates two intermediates with nonadjacent folded regions of the protein $\alpha_0 + \beta_4\alpha_4 + \alpha_5 + \alpha_6\beta_7$ (I_2) and $\alpha_0 + \beta_4$ – β_8 (I_1) and (40). These aldolase intermediates also demonstrate a preference for early C-terminal folding.

The differences in these pathways suggest that a high degree of structural diversity may exist within the TIM-barrel fold family which is not conveyed with the simple $(\beta\alpha)_8$ fold description. The folding mechanism must be determined by more subtle structural properties in the TIM-barrel proteins, such as 1), the slight differences in the contact topology due to different position and lengths of the α -helices and β -strands, or 2), differences in hydrophobic-residue packing within the barrel center. Although not addressed in this study, non-native contacts, proline isomerization, and disulphide formation can play an important role in TIM-barrel protein folding (47,51,52).

The primary question addressed in this work is whether the contacts determined from the x-ray crystal structure of γ TIM are by themselves sufficient to build an accurate protein folding model. An accurate γ TIM model, which correctly selects the number of folding intermediates and their structures, would support the hypothesis that contact topology is the primary determinant of γ TIM folding (2, 19,53). This would also support the conclusion that the energy landscape of γ TIM is highly funneled to the native state.

Previous computational TIM-barrel folding studies have investigated the folding of monomeric TIM barrels (16,54). For this study of γ TIM, the folding is complicated by a binding event that is necessary to form the native dimer state. Such a simulation involves simulating not just one large protein chain but two. Fortunately, minimalist Gō models, which approximate each amino acid with a single C_α atom, are uniquely suited to successfully simulate this large protein assembly system.

In this study, simulations were made to investigate both a monomeric model (one chain) and a dimer model (two chains) of γ TIM barrel folding in the context of kinetic protein folding and unfolding. To test the hypothesis that the energy landscape of γ TIM is funneled to the native state, the following questions were investigated:

1. Are the tertiary structures in progressive stages of folding similar to those of unfolding, and are these structures similar between the monomer and dimer models?
2. Are conformations in the stages of folding populated differently in simulations of monomer unfolding, dimer unfolding, monomer refolding, and dimer refolding?
3. Is complete folding of the γ TIM monomer required to form the dimer or can the two chains commit to a bound state before complete folding?
4. Do the structures and the basic pathway agree with the currently available experimental data?

With respect to question 4, a number of experiments have studied the equilibrium unfolding pathway of the γ TIM dimer. A number of global structural probes have indicated two-step equilibrium unfolding by chemical denaturants with a single monomeric intermediate ($\Delta G_{\text{NI}}^{\text{H}_2\text{O}} \approx 17$ kcal/mol, $\Delta G_{\text{IU}}^{\text{H}_2\text{O}} \approx 4$ kcal/mol) (33,55,56). Also, a residue-specific misincorporation proton alkyl exchange (MPAX) study has indicated a three-step unfolding mechanism with two intermediates ($\Delta G_{\text{NI}_1}^{\text{H}_2\text{O}} \approx 5$ kcal/mol, $\Delta G_{\text{I}_1\text{I}_2}^{\text{H}_2\text{O}} \approx 5$ kcal/mol, $\Delta G_{\text{I}_2\text{U}}^{\text{H}_2\text{O}} \approx 4$ kcal/mol) (34). This MPAX study provides detailed structural information on dominant intermediates in the folding ensemble of γ TIM but did not determine whether these intermediates were monomeric or dimeric. By comparing this theoretical study to these experiments, this work provides a complementary perspective on how the energy landscape of γ TIM guides its folding to the native state.

MATERIALS AND METHODS

Molecular dynamics

Molecular dynamics (MD) simulations were carried out using AMBER 6 software, compiled on a Linux platform, employing the sander_classic program as an integrator for initial energy minimization and subsequent molecular dynamics (57). The following describes the AMBER sander_classic molecular dynamics parameters used in this study. The specific parameter values are listed in parentheses. The time step was 0.001 ps (DT = 0.001). Translational and rotational motion was removed at the beginning of each run and every 1000 time steps thereafter (NTCM = 1, NSCM = 1000, NDFMIN = 0). Initial velocities were randomly selected (INIT = 3, IG = random). If the absolute value of the velocity of any atom exceeded 500 Å/time step, velocities were scaled such that the absolute value of the velocity of that atom was 500 Å/time step (VLIMIT = 500). Temperature was maintained with external bath using the method of Berendsen (58), with a coupling constant of 0.2 ps (NTT = 5, TAUTP = 0.2, TAUTS = 0.2). If the simulation temperature T_{sim} exceeded the average temperature T by >10 K, velocities were scaled such that $T_{\text{sim}} = T$. SHAKE was not used. Although no electrostatics were involved in the molecular dynamics, a default constant dielectric was used (IDIEL = 1) with a default dielectric constant of 1 (DIELC = 1). The particle mesh Ewald method was not used (IEWALD = 0). During each integration step, interactions between all atom pairs were calculated and this contact pairlist was updated only once at the beginning of the simulation (CUT = 9999, NSNB = 9999). No periodic boundary or pressure regulation was used (NTB = 0, NTP = 0). Structures and energies were saved every 1.5 ps (NTPR = 1500, NTWR = 1500, NTWX = 1500, NTWV = 1500, NTWE = 1500).

For dimer simulations, two separate protein chains were simulated. To prevent the chains from flying apart, a weak harmonic term was placed on the center of mass of each protein chain shown below in Eq. 1 (59,60):

$$R = k(D - D_0)^2. \quad (1)$$

In Eq. 1, k is the spring constant (0.01 kcal/mol), D is the angstrom distance between the center of masses of the two protein chains at a given point in the simulation, and $D_0 = 7.6$ Å (this is the distance between the two centers of mass in the reduced dimer model, which is equivalent to 29 Å in the real protein dimer). This value of $k = 0.01$ kcal/mol permits complete physical separation, with free rotational freedom, of the unbound protein chains. For dimer-unfolding trajectories, lower values of k yielded essentially the same results as those with $k = 0.01$ (data not shown). For dimer refolding trajectories, lower values of k yielded a similar protein folding pathway as those with $k = 0.01$. However, the rate of binding of the two γ TIM chains is slowed as k is decreased, so that fewer trajectories successfully reach the native dimer state during the simulation timeframe. The spring constant of $k = 0.01$ kcal/mol was the best compromise between approximating a real (i.e., more dilute) protein concentration and permitting enough simulations to successfully refold. The magnitude of k in this study is consistent with harmonic restraints used in previous studies (59,60).

Gō model

The PDB structure used to build the Gō model is 1YPI (Fig. 1). Fig. 1 shows that, although the individual monomers are virtually identical and the interface involves residues at the N-terminus of the protein, the structure is not a symmetric dimer, since the two monomers (blue, monomer A; and red, monomer B) do not orient as a mirror image of one another. As shown in Fig. 1, the two monomers orient with residues on the side of each barrel, but the barrel axes of each monomer are oriented at $\sim 90^\circ$ to one another. In Fig. 1, the dimer interface residues are shown in cyan for monomer A and in yellow for monomer B. Residues at the dimer interface are defined as having at least one atom in contact with the other chain and include residues 10 (β_1), 12–17 (β_1 - α_1 loop), 43–46 (β_2 - α_2 loop), 48 (α_2), 64–67 + 69–79 (β_3 - α_3 loop), 82–83 + 85–86 (α_3), 92 (β_4), and 95 + 97–98 + 101–102 + 108 (α_4).

To model γ TIM, each amino acid is approximated with its single backbone C_α atom as shown in Fig. 2. To facilitate faster MD sampling, a reduced protein model was used in which the distance between protein atoms was reduced 3.8-fold so that the C_α - C_α bond length was equal to 1 Å. This 1-Å bond-length model was shown to give identical folding behavior to protein models with 3.8 Å bond length, with a twofold increase in sampling efficiency (data not shown). This modified model helped with the simulation of the large γ TIM protein system. The overall potential energy for a given protein conformation is given by Eq. 2:

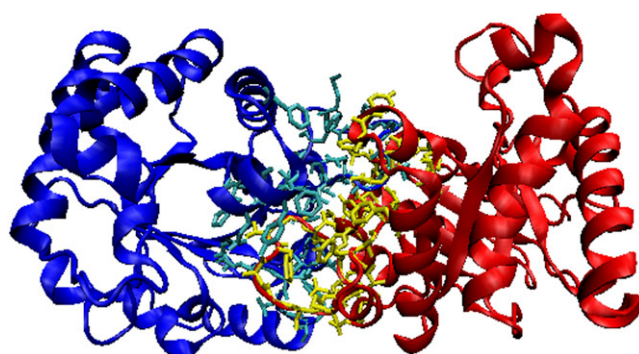


FIGURE 1 Triosephosphate isomerase dimer from PDB structure 1YPI. One monomer is colored blue with interface residues in cyan. The other monomer is colored red with interface residues in yellow.

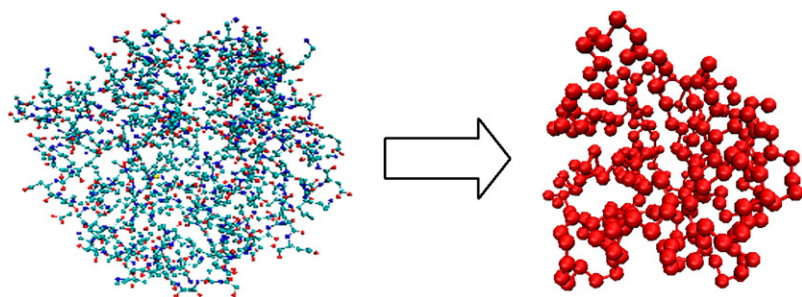


FIGURE 2 All atom coordinates (*left*) and C_α model (*right*) of the γ TIM monomer.

$$E_{\text{total}} = E_{\text{bond}} + E_{\text{angle}} + E_{\text{dihedral}} + E_{\text{LJ}} + E_{\text{rep}}. \quad (2)$$

Consistent with the original Gō model (26), the minimum energy of each energy term is obtained when the protein is in the native folded state. The parameters used in this study were selected because they had produced an accurate folding temperature and stability of chymotrypsin inhibitor 2 and tryptophan synthase in previous work (61).

For covalent bond distance terms,

$$E_{\text{bond}} = \sum_{\text{bonds}} \frac{1}{2} \epsilon_r (r - r_0)^2, \quad (3)$$

where $\epsilon_r = 100 \text{ kcal mol}^{-1} \text{ \AA}^{-2}$ is the bond energy, r is the bond distance in the simulation, and r_0 is the native $C_\alpha C_\alpha$ bond distance in the reduced C_α PDB structure, summed over all bonds in the reduced C_α PDB structure.

For the bond angle term,

$$E_{\text{angle}} = \sum_{\text{angles}} \frac{1}{2} \epsilon_\theta (\theta - \theta_0)^2, \quad (4)$$

where $\epsilon_\theta = 20 \text{ kcal mol}^{-1} \text{ deg}^{-2}$ is the bond angle energy, θ is the bond angle in the simulation, and θ_0 is the $C_\alpha C_\alpha C_\alpha$ native bond angle, summed over all bond angles in the C_α PDB structure.

For dihedral energies,

$$E_{\text{dihedral}} = \sum_{\text{dihedrals}} \left[\epsilon_\phi^1 [1 - \cos(\phi - \phi_0)] + \epsilon_\phi^2 [1 - \cos(3(\phi - \phi_0))] \right], \quad (5)$$

where $\epsilon_\phi^1 = 0.8 \text{ kcal/mol}$, $\epsilon_\phi^2 = 0.4 \text{ kcal/mol}$, ϕ is the dihedral angle in the simulation, and ϕ_0 is the $C_\alpha C_\alpha C_\alpha C_\alpha$ native dihedral angle in the reduced C_α PDB structure, summed over all dihedral angles in the C_α PDB structure.

In the Gō model, two C_α atoms in a protein were selected as attractive if they were separated by four or more residues and were indicated to be in contact using contacts-of structural-units (CSU) analysis (62). Each attractive C_α - C_α contact is described by an attractive Lennard-Jones potential:

$$E_{\text{LJ}} = \sum_{|i-j| \geq 3} \epsilon_{\text{LJ}} \left[5 \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - 6 \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{10} \right], \quad (6)$$

where $\epsilon_{\text{LJ}} = 0.8 \text{ kcal/mol}$ is the contact energy; σ_{ij} is the native distance between the two contact atoms, i and j , given from the crystal structure; and r_{ij} is the distance between the two contact atoms, i and j , determined for a given iteration of the simulation.

For dimer models, if an intermolecular contact between residue i on chain A and residue j on chain B was determined to exist, it received a contact energy of $\epsilon_{\text{LJ}} = 0.8 \text{ kcal/mol}$ and native distance parameter (σ_{ij}) equal to the native distance between the two contact atoms, i (in chain A) and j (in chain

B). In the rare event where residues i and j both form an intramolecular (tertiary) contact and an intermolecular (quaternary) contact, the native distance parameter (σ_{ij}) is set to the intramolecular (tertiary) native-state distance.

If any two atoms are not determined to be attractive or fall within three residues of each other ($i, i + 3$), then their interaction was defined by a repulsive term:

$$E_{\text{rep}} = \sum_{i,j} \epsilon_{\text{rep}} \left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12}, \quad (7)$$

where $\epsilon_{\text{rep}} = 0.8 \text{ kcal/mol}$ is the repulsive energy, σ_{ij} is half the hard-sphere distance between two repulsive atoms i and j (1.9 \AA), and r_{ij} is the distance between the two repulsive atoms, i and j , determined for a given iteration of the simulation.

Kinetic simulations

Four types of kinetic simulations are conducted with γ TIM: 1), monomer unfolding, 2), monomer refolding, 3), dimer unfolding, and 4), dimer refolding. A schematic of these four simulation types is shown in Fig. 3. For unfolding simulations of γ TIM monomers and dimers, 30 kinetic trajectories are collected to obtain statistically significant measurements. For refolding simulations of γ TIM monomers, 30 kinetic trajectories are also collected to obtain statistically significant measurements. For refolding simulations of γ TIM dimers, 90 kinetic trajectories are collected to obtain statistically significant measurements.

The initial coordinates used for MD simulations of γ TIM were obtained from simulated annealing, using the 1YPI protein data bank (PDB) coordinates as an initial structure (Fig. 1). For unfolding simulations, the starting coordinates of each refolding trajectory are obtained from the final structure of a short simulation at 330 K of a randomly determined length (500–1500 ps) and random initial velocities. These starting 330 K coordinates are immediately placed into a 420 K simulation temperature with random initial velocities and run for 5 ns (5×10^6 time steps). For refolding simulations, the starting coordinates of each refolding trajectory are obtained from the final structure of a short simulation at 999 K of a randomly determined length (500–1500 ps) and random initial velocities, followed by 100 ps at 420 K and random initial velocities. These starting 420 K coordinates are immediately placed into a 330 K simulation temperature with random initial velocities and run for 20 ns (20×10^6 time steps).

All simulations were performed using molecular dynamics from randomized initial structures and velocities. This procedure provides a degree of variability between the behavior of each kinetic trajectory. However, the MD trajectories themselves are deterministic after the initial structure and velocity conditions are applied. Thus, one might expect the results presented here to differ from those of Langevin dynamics simulations, in which random forces are continually applied so as to mimic the ongoing steric interactions from collisions between the protein atoms and the solvent atoms. To a certain degree, the Gō model does implicitly account for the energetic

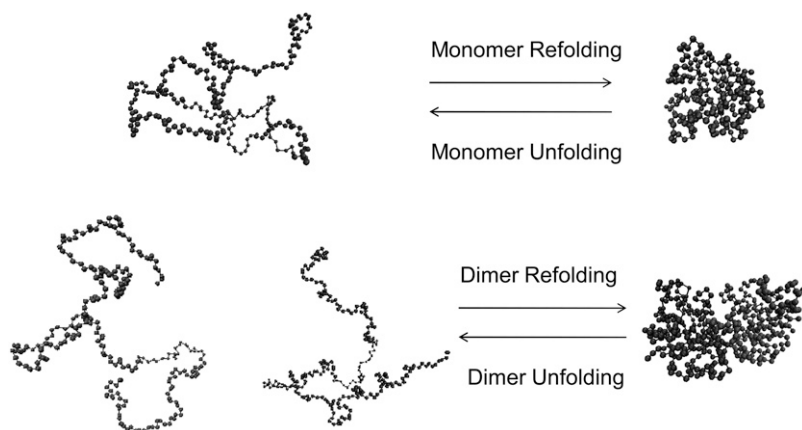


FIGURE 3 Schematic for folding (right arrows) and unfolding (left arrows) for (top) γ TIM Gō model monomer and (bottom) γ TIM Gō model dimer.

interactions between atoms of the solvent and atoms of the protein, particularly hydrophobic interactions. However, the random steric interactions of Langevin dynamics simulations are not included when MD is used to study the C_α Gō model. This study neglects these steric interactions to explore the extent to which the γ TIM folding pathway is guided through deterministic interactions. Experimental features, such as populated intermediate states, of the γ TIM kinetic folding pathway that are not captured by MD simulations may highlight the importance of stochastic events in populating such states.

For unfolding trajectories, 5 ns of simulation time at 420 K was sufficient to completely unfold all 30 monomer and 30 dimer γ TIM proteins in the simulations. Complete unfolding was determined by total contact energies E_{LJ} and values of Q close to 0 (typically $E_{LJ} \approx -30$ kcal/mol and $Q \approx 0$ for an unfolded γ TIM chain with 587 contacts). These values of E_{LJ} and Q are consistent with the infrequent interactions inherent in a randomly moving freely jointed chain (data not shown). For refolding trajectories of the monomer γ TIM protein model, 20 ns of simulation time at 330 K successfully refolded 90% of the simulations. For refolding trajectories of the dimer γ TIM protein model, 20 ns of simulation time at 330 K successfully refolded 32% of the simulations. All refolding trajectories that did not refold within 20 ns did not refold after an additional 10 ns of simulation, indicating that they were trapped in a misfolded state, requiring extensive simulation time to escape. As such, the data from incomplete simulations were not included in the data analysis. Each simulation condition studied—monomer unfolding (30 trajectories, 100% successful), monomer refolding (30 trajectories, 90% successful), dimer unfolding (30 trajectories, 100% successful), and dimer refolding (90 trajectories, 32% successful)—produced ~ 30 successful trajectories, which were used for analysis.

Statistical errors reported throughout this article are based on the following grouping of the kinetic trajectories. The trajectories are divided into three groups: 1), trajectories 1–10, 11–20, and 21–30 for monomer unfolding, monomer refolding, and dimer unfolding; 2), trajectories 1–30, 31–60, and 61–90 for dimer refolding. Within each group are ~ 10 successfully refolded or unfolded trajectories. Properties of each group are averaged and these three separate averages are used to determine a global average and standard deviation. The data reported in this study are the averages of three separate analyses of each group. The errors shown in the figures are the standard deviations of each data point.

Simulation analysis

Throughout each 5-ns unfolding simulation, 3330 structures were obtained (one structure every 1.5 ps). Throughout each 20-ns refolding simulation, 13,320 structures were obtained (one structure every 1.5 ps). For each structure sampled throughout the simulations, the total number of native contacts (Q) formed was calculated according to where each native contact

was determined to be formed if it fell within 1.5 times the native distance. The maximum number of tertiary native contacts (folding contacts within the monomer) possible for each monomer simulation was 587. The maximum number of tertiary native contacts possible for each dimer simulation was 1174, twice that of the monomer. The maximum number of intermolecular native contacts (binding contacts at the dimer interface between the two monomers) was 108.

In theory, the distance cutoff for contacts can be of any value as long as different structural states (with different degrees of folding) can be reasonably classified as different values of Q (or another order parameter, such as P_{fold}). For Gō models, similar folding mechanisms have been demonstrated during separate studies of chymotrypsin inhibitor 2 using different contact cutoff values of 1.2 (53) and 1.5 (61). A distance cutoff is only problematic when an excessive population of Q is found at $Q = 0$ (cutoff is too small) or at $Q = Q_{\text{max}}$ (cutoff is too large), where significantly different structures are placed in the same Q bin. Prior analysis has shown that the changes in the cutoff range between 1.2 and 1.6 times the native distance does not alter the interpretation of protein folding pathways, although the Q distribution width of native, intermediate, and unfolded populations will indeed change slightly (data not shown). As was done in previous studies, this study uses a contact cutoff of $1.5 \times$ (native distance) to produce a distribution of Q states that best approximates the relative distribution widths of E_{LJ} contact energies between the native and unfolded ensembles (16,61). Using this cutoff of 1.5, no structures in the simulations ever occupied values of Q at either $Q = 0$ or $Q = Q_{\text{max}}$, indicating that the density of states was adequately binned in the intermediate values of Q between 0 and Q_{max} .

For each simulation condition studied, the probability that the simulation structure had a value of $Q = i$ was computed through Eqs. 8a and 8b:

$$\text{Probability}(Q = i) = \frac{\sum_{n=1}^N \sum_{t=1}^T \delta_{Q(n,t),i}}{NT}, \quad (8a)$$

where

$$\delta_{Q(n,t),i} = \begin{cases} 1, & \text{if } Q(n,t) = i \\ 0, & \text{if } Q(n,t) \neq i \end{cases} \quad (8b)$$

In Eq. 8a, N is the total number of simulations in each group (10 maximum) and T is the length of each simulation (3330 for unfolding, 13,320 for refolding). For intramolecular contacts within each γ TIM protein chain, i is varied between 0 and 587 for monomer simulations and between 0 and 1174 for dimer simulations. For intermolecular contacts at the dimer interface, i is varied between 0 and 108.

To identify the structures of intermediate stages of folding, the simulation structures were grouped by similar values of Q tertiary contacts. Group A consists of structures where $Q = 0$ –100 for the monomer and 0–200 for the

dimer. Group B consists of structures where $Q = 101$ –200 for the monomer and 201–400 for the dimer. Group C consists of structures where $Q = 201$ –300 for the monomer and 401–600 for the dimer. Group D consists of structures where $Q = 301$ –400 for the monomer and 601–800 for the dimer. Group E consists of structures where $Q = 401$ –500 for the monomer and 801–1000 for the dimer. Group F consists of structures where $Q = 501$ –587 for the monomer and 1001–1174 for the dimer. Within groups A–F, the probability that each residue i is folded is calculated through Eqs. 9a and 9b:

$$\text{Probability Folded } (i) = \frac{\sum_{n=1}^N \sum_{t=1}^T \sum_{p=0}^P \delta_{D(i,n,t,p), D(i,X,p)}}{NTP}, \quad (9a)$$

where

$$\delta_{jk} = \begin{cases} 1, & \text{if } D(i, n, t, p) \leq 1.5 * D(i, X, p) \\ 0, & \text{if } D(i, n, t, p) > 1.5 * D(i, X, p) \end{cases} \quad (9b)$$

In Eq. 9a, N is the total number of simulations in each group (10 maximum), T is the length of each simulation (3330 for unfolding, 13,320 for refolding), P is the total number of contact pairs involving residue i (varies from 0 to 16), $D(i, n, t, p)$ is the distance of C_α atoms of residue i and contact partner, defined by p , in the simulation at a given set of n, t values, and $D(i, X, p)$ is the distance of C_α atoms of residue i and contact partner, defined by p , in the reduced PDB structure 1YPI (denoted as X). In the case of dimer simulations, the value reported is the average value of Probability Folded (i) from each of the two monomer chains. For residues in γ TIM, i is varied between 1 and 247.

RESULTS

Fig. 4 shows representative trajectories of dimer unfolding (Fig. 4 A) and dimer refolding (Fig. 4 B) of γ TIM. Fig. 4 A plots the total number of intramolecular native contacts (Q) within each monomer (black lines) and intermolecular native contacts at the dimer interface (gray lines) as a function of time steps in a simulation starting from a native dimer conformation and unfolding at 420 K. Fig. 4 B plots the total number of intramolecular Q within each monomer (black lines) and intermolecular Q at the dimer interface (gray lines) as a function of time steps in a simulation starting from an unfolded monomer conformation and refolding at 330 K. These simulations are representative of the 30 total simulations acquired for each simulation type—monomer unfolding, monomer refolding, dimer unfolding, and dimer refolding. Although Fig. 4, A and B, is representative of many of the other trajectories, it is important to note that each trajectory samples its own unique set of intermediate states during unfolding or refolding. Simulation trajectories of monomer unfolding and refolding appear similar to the dimer intramolecular Q trajectories (black lines) in Fig. 4, A and B, albeit with slightly different intermediate folding stages populated.

Structures from the trajectories can be grouped based on which stage of folding they fall into (i.e., Q). The fraction of each residue that is folded can be quantified for structures within each range of Q . As discussed in Materials and Methods, the structures in the trajectories fall into six folding “groups”, A–F. Group A is the least folded and includes

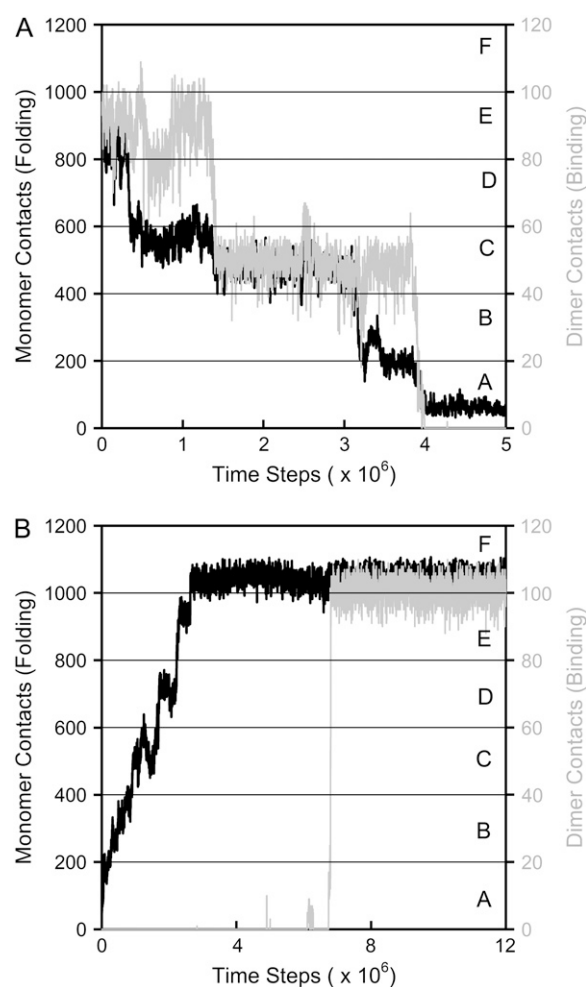


FIGURE 4 Sample trajectories of γ TIM dimer (A) unfolding and (B) refolding. The left y axis shows the total number of intramolecular contacts (black lines) for the two protein chains (587 maximum for each chain = 1174 total). The right y axis shows the number of intermolecular contacts (gray lines) at the dimer interface between the two chains (108 maximum).

largely unfolded structures. Group F is the most folded and includes largely native structures. Groups B–E represent intermediate stages of folding with increasing values of Q . The values of Q used to define groups A–F only involve intramolecular contacts within monomer chains and do not include dimer contacts at the interface.

Fig. 5 shows the degree of folding of each γ TIM residue in groups B–E. Fig. 5 A highlights the regions of secondary structure throughout the γ TIM monomer. Fig. 5, B–E, shows the fractions folded of each residue for groups B–E, respectively. Since these groups are sampled through the four different simulation types, all four are shown for comparison: monomer unfolding (dashed gray lines), monomer refolding (solid gray lines), dimer unfolding (dashed black lines), and dimer refolding (solid black lines). With minimal exception, all simulation types produced largely similar results. Fig. 5 B shows that residues within regions

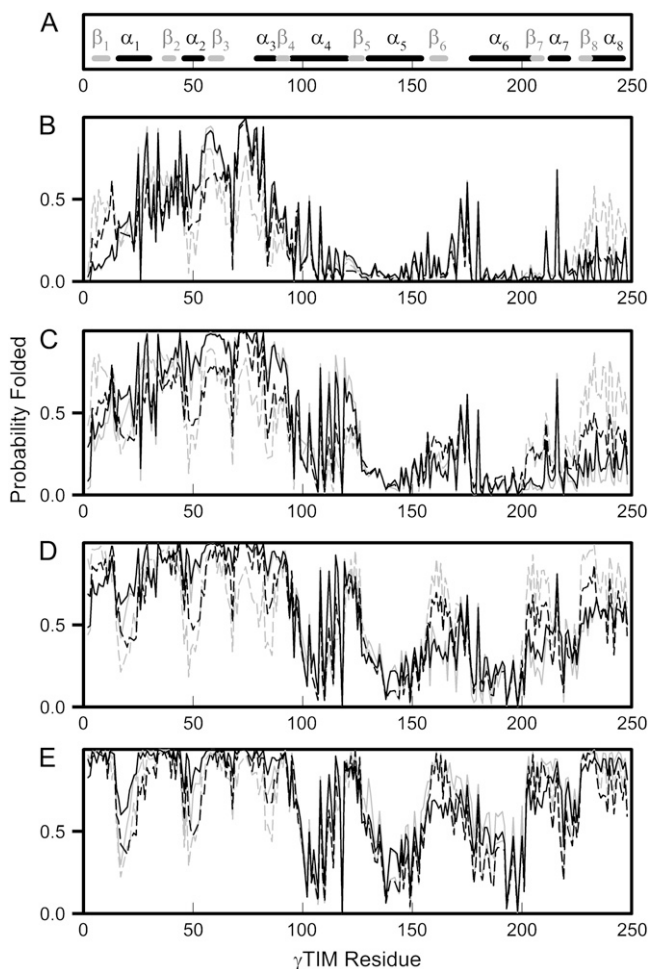


FIGURE 5 Intermediate stages of γ TIM monomer unfolding (dashed gray lines), dimer unfolding (dashed black lines), monomer refolding (solid gray lines), and dimer refolding (solid black lines). (A) α -helix (black) and β -sheet (gray) regions of γ TIM. (B) Probability of residue folding in group B: β_2 – β_3 . (C) Probability of residue folding in group C: β_2 – β_4 . (D) Probability of residue folding in group D: β_1 – β_5 . (E) Probability of residue folding in group E: β_1 – β_8 .

β_2 – β_3 are the initial regions to fold in group B. Fig. 5 C shows that folding has increased to involve residues within regions β_2 – β_4 in group C. Fig. 5 D shows that folding has increased to residues within regions β_1 – β_5 in group D. Fig. 5 E shows that folding increased to residues within regions β_1 – β_8 in group E, with some residues in α -helical regions remaining unfolded. Residues in group A (not shown) are mostly unfolded and residues in group F (also not shown) are mostly folded.

The results shown in Fig. 5, A–E, indicate that the simulation type (monomer unfolding, dimer refolding, etc.) does not significantly change the folding order of residues within the protein. However, the probability of populating groups B–E does depend on the simulation type. Fig. 6, A–D, shows the probability that structures with each value of Q will be populated during the course of the simulation. Fig. 6

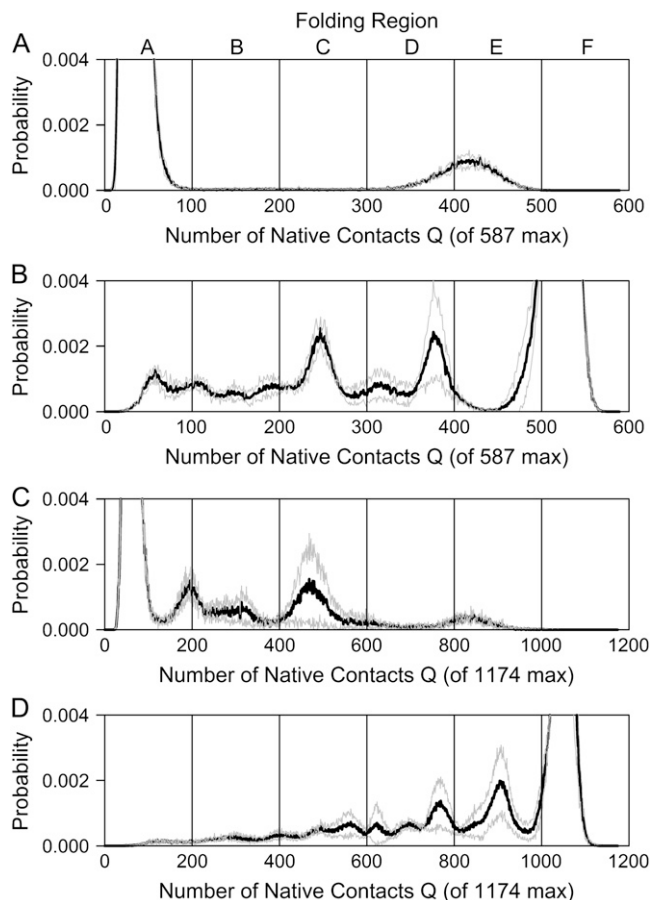


FIGURE 6 (A) Probability of populating folding groups A–F during monomer unfolding, (B) monomer refolding, (C) dimer unfolding, and (D) dimer refolding. Error boundaries are indicated in gray.

A shows that the probability of finding intermediate values of Q between the unfolded ensemble in group A and the folded ensemble in group E is very low, indicating that no intermediates exist in monomer unfolding. Fig. 6 B shows two Q -probability peaks in groups C and D, indicating that two intermediates exist in monomer refolding simulations. Fig. 6 C shows two Q -probability peaks, one between groups A and B and the other in group C, indicating that two intermediates exist in dimer-unfolding simulations. Fig. 6 D shows two Q -probability peaks, in groups D and E, indicating that two intermediates exist in dimer refolding simulations. It should be noted that the “folded” ensemble during unfolding simulations falls in group E (Fig. 6, A and C) instead of group F (Fig. 6, B and D). This is because the increased temperature of unfolding simulations makes the weaker contacts of the folded structure less likely to be formed, producing a lower value of Q for the folded state.

Fig. 7, A and B, shows the average number of intramolecular (monomer) contacts and intermolecular (dimer-interface) contacts that disappear during unfolding (Fig. 7 A) or accumulate during refolding (Fig. 7 B). Fig. 7, A and B, only applies to simulations of the dimer Gō models of γ TIM,

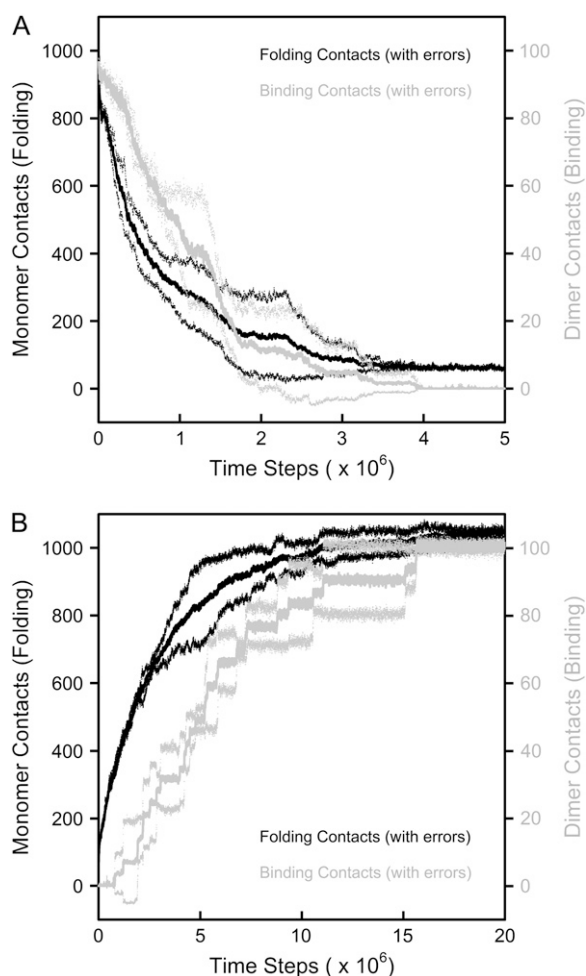


FIGURE 7 Average number of intramolecular (black lines) or intermolecular (gray lines) contacts obtained from 30 trajectories of (A) dimer unfolding and (B) dimer refolding. Error boundaries are indicated with black points for intramolecular contacts and gray points for intermolecular contacts.

since monomer simulations do not have intermolecular contacts. For dimer unfolding in Fig. 7 A, it can be seen that, on average, contacts associated with unfolding (monomer contacts) decrease at a similar rate to contacts associated with unbinding (dimer contacts). This finding suggests that dimer unbinding occurs at the same time that the monomers unfold, within the experimental error of the simulations. For dimer refolding in Fig. 7 B, it can be seen that, on average, contacts associated with folding (monomer contacts) increase at a faster rate than contacts associated with binding (dimer contacts). This finding is within the experimental error of the simulations and suggests that a significant amount of folding precedes dimer formation.

Table 1 gives a different presentation of the γ TIM binding versus folding data shown in Fig. 7, A and B. Table 1 indicates, for each folding group, the number of dimer-unfolding simulations in which unbinding occurs (*middle column*) and the number of dimer-refolding simulations in

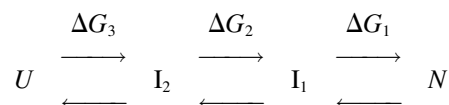
TABLE 1 Folding groups where binding or unbinding occurs in dimer simulations

Folding group	Number of dimer unfolding simulations where unbinding occurs	Number of dimer refolding simulations where binding occurs
A	30 (100%)	0 (0%)
B	0 (0%)	1 (3%)
C	0 (0%)	8 (27%)
D	0 (0%)	10 (33%)
E	0 (0%)	6 (20%)
F	0 (0%)	5 (17%)

which binding occurs (*right-hand column*). In Table 1, an unbinding event occurs when the number of intermolecular contacts between the two γ TIM protein chains falls below 20 and a binding event occurs when the number of intermolecular contacts between the two γ TIM protein chains increases to >20 . The number 20 was arbitrarily selected to ensure that the two γ TIM protein chains were bound at a level of contact above random collisions between two unfolded chains.

For dimer unfolding, Table 1 reinforces the data in Fig. 7 A by demonstrating that unbinding never completely occurs until the γ TIM protein chains completely unfold in group A (*middle column*). In contrast, for dimer refolding, Table 1 shows that binding can occur in a number of different folding groups. From Table 1, it is clear that no binding ever occurs directly from the unfolded ensembles in group A (through a “fly-casting” mechanism) and only occurs once folding has led to group B (17). Therefore, some folding must occur to facilitate binding. However, once the dimer interface residues between β_2 and β_3 of group B have folded, binding can readily occur in later stages of folding (groups C–F). Therefore, the intermediate populations of groups B–E in dimer refolding consist of a mixture of monomer and dimer oligomeric states.

Fig. 8 displays previously published experimental data on the folding/unfolding of γ TIM using MPAX experiments (34). The MPAX experiment measures the equilibrium constant between the unfolded (assumed to be alkyl-exchangeable) and folded (assumed to be unexchangeable) states of individual amino acids in the γ TIM protein under nativelike conditions (low-denaturant concentrations) (63). It was found that the stability of the γ TIM residues fell into three classes: low ($\Delta G_1 \approx 3.7$ kcal/mol), medium ($\Delta G_2 \approx 6.5$ kcal/mol), and high ($\Delta G_3 \approx 8.5$ kcal/mol) (34). Since the rates of iodoacetamide incorporation obey a first-order rate for all residues studied, it was concluded that the sequential model below was the most appropriate (34):



Although it is possible that one or both of the intermediates might be off-pathway, the spatial clustering of medium- and

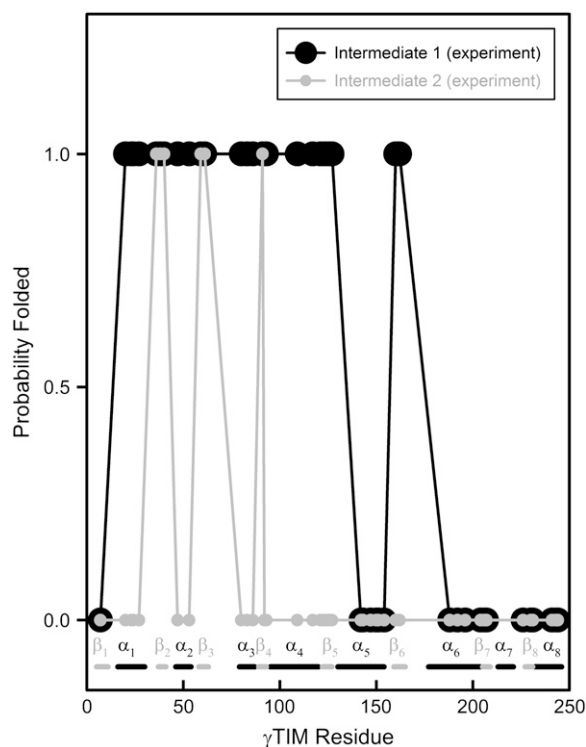


FIGURE 8 Residues folded in early-folding intermediate 2 (gray points) and late-folding intermediate 1 (black points) for 47 residues studied with alkyl-exchange experiments.

high-stability residues in the γ TIM chain (shown in Fig. 8) suggests that the above model is reasonable.

Assuming that the above model is correct, the structures of intermediates I_2 and I_1 can be deduced from Fig. 8. In Fig. 8, residues of intermediate I_2 are indicated in red and residues of intermediate I_1 in blue. Residues are assumed to be either “folded” or “unfolded” based on the MPAX study. This large set of experimental data offers a unique opportunity to evaluate whether the folding pathways predicted by simulations in this study are correct.

Different experimental methods do not measure the properties of intermediate states with the same sensitivity (64). For example, an MPAX experiment may not detect the same number of intermediates as NMR peak shifts. Likewise, properties measured by simulation, such as Q , may not be directly proportional with alkyl-exchange propensity in the MPAX experiment. To avoid such complications, this study uses a simplistic approach to evaluate whether a residue is folded or unfolded in a simulation:

Probability > 0.5 = folded (value = 1).

Probability < 0.5 = unfolded (value = 0).

In a sense, the simulation must guess 0 or 1 for each of the 47 residues studied with MPAX in Fig. 8. For these 47 residues, simulation guesses will be evaluated against the “real” 0 or 1 values determined from Fig. 8 for intermediates I_2 and I_1 .

For a complete analysis, all folding groups, A–F, from the simulation were compared. Furthermore, the four simulation types (monomer unfolding, monomer refolding, dimer unfolding, and dimer refolding) were compared. In Fig. 9, A and B, the percentages of “correct” guesses for the four simulation types and six folding groups are shown in terms of how they compare to intermediates I_2 (Fig. 9 A) and I_1 (Fig. 9 B). When the simulations are compared to I_2 in Fig. 9 A, it is clear that the early folding groups, A–C, match very well with the experimental results, whereas the more folded groups, D–F, fit less well as the number of native contacts increases. All four simulation types match equally well within the error of the simulation method.

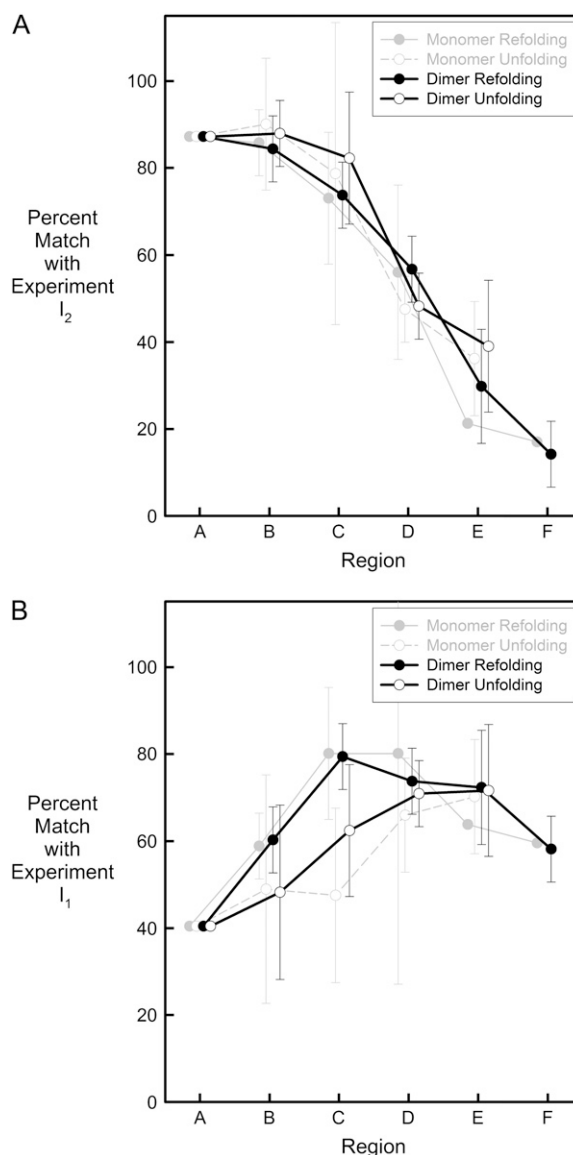


FIGURE 9 Percent match of simulation folding groups A–F (Fig. 5) at predicting experimental intermediates (A) 2 and (B) 1 from Fig. 8. Data points from simulations of monomer refolding, monomer unfolding, dimer refolding, and dimer unfolding are slightly offset to aid the eye.

When the simulations are compared to I_1 in Fig. 9 *B*, it is clear that partially folded groups C and D match best with the experimental results, whereas more-folded (groups E and F) and less-folded (groups A and B) groups fit less well. In Fig. 9 *B*, structures in refolding simulations match the structure of I_2 better than those of unfolding simulations in group C, the group having the greatest structural match with I_1 .

DISCUSSION

The goal of the research presented here is to provide answers to the following four questions:

1. Are the structures in partially folded stages during folding similar to those in equivalent stages during unfolding. Also, are these intermediate structures similar between simulations using monomer and dimer models?
2. Are conformations in the stages of folding populated differently in simulations of monomer unfolding, dimer unfolding, monomer refolding, and dimer refolding?
3. Is complete folding of the γ TIM monomer required for formation of the dimer, or can the two chains commit to a bound state before complete folding?
4. Do the structures and the basic pathway agree with the currently available experimental data?

In these simulations, the answers to the above questions are: 1), yes; 2), no; 3), no; and 4), yes. A further discussion of these questions follows.

Answer to question 1: Yes. The folding order of simulated γ TIM is essentially the same regardless of whether the simulation is monomer unfolding, monomer refolding, dimer unfolding, or dimer refolding.

Fig. 5, *A–E*, highlights the more structured residues of γ TIM in intermediate folding groups B (Fig. 5 *B*), C (Fig. 5 *C*), D (Fig. 5 *D*), and E (Fig. 5 *E*). Generally, the order of intramolecular structure formation (“folding order”) is similar between monomer unfolding, monomer refolding, dimer unfolding, and dimer refolding simulations. Within these four simulation possibilities, there does not seem to be any significant difference in folding order between monomer and dimer simulations. There are, however, slight differences in folding order between refolding and unfolding simulations. Unfolding simulations tend to have slightly lower residue folding probability in the β_2 – β_4 “core” than refolding simulations. Unfolding simulations also have slightly higher residue folding probability at the N- and C-termini than refolding simulations. These differences are most pronounced in group C (Fig. 5 *C*). Regardless of these differences, refolding and unfolding γ TIM simulation results remain very similar in Fig. 5, *B–E*.

There are both intellectual and practical implications of this finding. On a scientific level, the folding order of γ TIM protein chains appears to be highly robust, since unfolding appears to be a reverse of the refolding pathway in Fig. 5, *B–E*. In addition, the relative order in which intramolecular

contacts are made is independent of whether a single γ TIM chain is folded or whether two γ TIM chains are folded together. This finding implies that either 1), folding completely precedes binding and unbinding completely precedes unfolding; or 2), the dimer interface contacts do not significantly change the folding order. Fig. 7, *A* and *B*, and Table 1 demonstrate that the first possibility is not correct for the simulations in this study. Therefore, the second possibility is most likely correct. It can be observed from the early monomer unfolding and refolding shown in Fig. 5 *B* that the initial contacts formed are in strands β_2 – β_4 , which is also the site of the dimer interface. In dimer simulations, the relative stability of this β_2 – β_4 region would be expected to increase and it would form at earlier time points of the dimer folding process. However, since it is already the most stable folding region in monomeric γ TIM folding, the overall folding order of the secondary structure remains unchanged. In other words, if one stabilizes the part of the protein that folds first, that part will still fold first.

From a practical standpoint, it is interesting that monomer and dimer simulations have a similar folding order of secondary structure. A finding that monomers and oligomers fold in a similar order in other oligomeric TIM barrels may aid future genomic-level folding analysis of TIM barrels. For example, oligomeric folding is considerably more computationally expensive than monomeric folding. Oligomer folding also involves subjective protein-specific decisions by the researcher, such as the priority of interface contacts over intramolecular contacts and the restraint strength (Eq. 1), which can complicate automated simulation and analysis strategies. If monomeric folding simulations have the same folding order as oligomeric simulations, limiting the simulations to monomer folding/unfolding would lead to much more simple and efficient computational approaches.

Answer to question 2: No. The folding intermediates of simulated γ TIM are populated differently depending on whether the simulation is monomer unfolding, monomer refolding, dimer unfolding, or dimer refolding.

Fig. 5, *A–E*, demonstrates that the structures of intermediates in groups B–E generally do not change, whether the simulation is conducted as monomer unfolding, monomer refolding, dimer unfolding, or dimer refolding. However, Fig. 6, *A–D*, shows that the probability of populating intermediate groups depends on the type of simulation. For monomer unfolding, no intermediates are populated between the folded and unfolded ensembles (Fig. 6 *A*). Although all other simulations produced two probability peaks at intermediate stages of folding, no simulation type produced these two peaks in the same folding groups. In monomer refolding, the probability peaks were in groups C and D (Fig. 6 *B*). In dimer unfolding, the probability peaks were in groups A/B and C (Fig. 6 *C*). In dimer refolding, the probability peaks were in groups D and E (Fig. 6 *D*).

Although the temperature (330 K vs. 420 K) difference can account for differences between unfolding and refolding

simulations, the differences between monomer and dimer group probabilities cannot be explained by thermal stability differences. In the dimer-unfolding simulations, intermediates in groups A/B and C are stabilized, a situation which did not exist in monomer unfolding. In dimer-refolding simulations, intermediates in groups more likely to be involved in dimer formation are stabilized (groups D and E) over intermediate groups populated in monomer refolding (groups C and D). Thus, although the folding order shown in Fig. 5, *B–E*, remains constant in the four different simulation types, the stability of the intermediate groups *B–E*, shown in Fig. 6, *A–D*, respectively, is not as robust.

Answer to question 3: No. Complete folding of the γ TIM monomer is not required to form the dimer.

Fig. 7 A and Table 1 show that partial dimer unfolding can occur without breaking all contacts at the dimer interface. This is also directly observed in the sample trajectory in Fig. 4 A. In addition, Table 1 shows that binding can occur during dimer refolding in the partially folded intermediate groups *B–E*, although most binding occurs in groups *C–E*. Although there are some differences in dimerized states between the groups in the unfolding and refolding simulations, partially unfolded dimers clearly exist in both unfolding and refolding simulations. In dimer unfolding, partially folded dimers appear to be obligatory, since dimer dissociation occurs in group A only when complete unfolding has taken place (Table 1, *middle column*). However, in dimer refolding, partially folded dimers do not seem to be obligatory, since five refolding trajectories completely fold into group F before dimerization (Table 1, *right-hand column*).

In the simulations, the reason for this is straightforward. Late unfolding and early refolding of residues in groups B and C occurs in the dimer interface region β_2 – β_4 (Fig. 5, *B* and *C*). In refolding, once this β_2 – β_4 region is folded, binding can occur. In unfolding, this β_2 – β_4 region must completely unfold to release the two bound protein chains. Although this can explain the γ TIM unfolding and folding pathways in the simulations, experiments have not shown any evidence of dimeric intermediates in γ TIM folding (33,55,56).

In terms of experimental comparison, equilibrium unfolding experiments do not support a γ TIM folding mechanism involving a partially unfolded dimeric intermediate (33,55,56). Instead, the proposed model of these studies involves a single partially unfolded monomeric intermediate (33,55,56). The experimental evidence for the monomeric intermediate rests in a decrease in hydrodynamic radius between the native state and the intermediate state and also through data fitting of multiple global spectroscopic probes (33,55,56).

In light of these experimental results, it would seem that dimeric intermediates in the present simulation studies are not physically realistic. However, for a number of reasons, the jury remains out as to whether the experiments and simulations of γ TIM agree or disagree on dimeric intermediates.

1. The simulations described here are conducted under kinetic conditions and the experiments are conducted under equilibrium conditions. Due to the size of the γ TIM protein system, equilibrium simulations were too time-consuming to be conducted in a reasonable amount of time for the project, even using the simplified Gō model in this study (16,65). To address γ TIM folding, the simulations were conducted as “kinetic experiments”, involving both thermal unfolding and refolding (16). These experiments, which have identified monomeric intermediates, were conducted under equilibrium conditions (33,55,56). Therefore, these studies may not be completely appropriate for a rigorous comparison with the simulations presented here.

Unfolding and refolding kinetic studies of γ TIM have also been conducted and also support the presence of intermediates in γ TIM folding and unfolding (32,55,66). However, the oligomeric nature of these kinetic intermediates is not known. Future kinetic studies of γ TIM may demonstrate the presence of dimeric intermediates.

2. Simulations are conducted under high protein concentration. For the dimer simulations to refold in a reasonable amount of simulation time, a relatively tight restraint must be employed (Eq. 1). The average distance between the center of mass of the two unfolded monomers in the simulations was ~ 80 Å, which implies a highly concentrated protein solution. In the simulations, this will promote binding at earlier times in refolding than would occur in experiments. Under the more dilute conditions of the experiments, the simulations will favor monomer folding before dimerization (33,55,56). Alternatively, if experiments (0.001–1.0% protein) were able to push the γ TIM concentration near that of the simulations (20–30% protein), it might be possible to force the γ TIM folding energy landscape to favor a dimeric intermediate state.

One caveat to this explanation lies in the kinetic simulations of dimer unfolding. Although equilibrium and kinetic refolding of γ TIM will be affected by γ TIM concentration, γ TIM dimer unfolding will not. Therefore, the presence of dimeric intermediates in γ TIM kinetic unfolding simulations cannot be explained by the effective protein concentration of the simulation. Future kinetic unfolding experiments of γ TIM are needed to verify this finding.

3. Dimeric intermediates may exist in equilibrium experiments. All the equilibrium folding experiments use global spectroscopic probes that measure average properties of the γ TIM protein (33,55,56). The studies argue for a monomeric intermediate through data fitting and measurements of hydrodynamic radius, but do not establish a homogeneous monomeric intermediate population (33,55,56). Although it is true that these average properties support the presence of some monomeric intermediates, it has not been confirmed that this ensemble consists of

100% monomeric intermediates. Future studies are necessary to resolve this issue more clearly.

Answer to question 4: Yes. The folding order matches well between simulations and MPAX experiments.

At this point, it should be noted that the MPAX study (34) contrasts slightly with other protein folding studies of γ TIM (33,55,56). The MPAX study identifies two equilibrium intermediates (34), whereas the other studies only identify one intermediate (33,55,56). This discrepancy can be accounted for by the nature of the experiments. MPAX measures the stability of intermediates under nativelike conditions (<1 M Gdn-HCl), whereas the other folding studies measure the stability of intermediates near the unfolding transition midpoint (1–2 M Gdn-HCl). Using the m -values, extrapolation of the ΔG of the two MPAX intermediates indicates that their stability is very similar under the conditions of the other folding studies ($\Delta G_{IU} \approx 1$ kcal/mol at 1.5 M Gdn-HCl for the C41V/C126A variant) (34). Therefore, the 1.5 M Gdn-HCl ensemble may appear as a single intermediate when measured with global structural probes such as fluorescence, circular dichroism, and size-exclusion chromatography. For purposes of the following simulation-experiment comparison, it is assumed that the γ TIM folding pathway consists of the two intermediates I_2 and I_1 identified with MPAX experiments (34).

Fig. 9, A and B, shows that the folding simulations have the potential of being highly predictive of the MPAX experimental intermediates I_2 and I_1 in Fig. 8. In groups A–F, which match best, the agreement is $>80\%$ in Fig. 9, A (groups A and B) and B (groups C and D).

The fact that the simulations match the experimental intermediate structures in some of groups A–F is a first test of the simulations. The second test is whether the highly populated groups in Fig. 6, A–D, are those that match well with the experiments. Table 2 shows which of groups A–F are highly populated in each simulation type and their match with the MPAX experiments. The least folded group identified with simulations is matched with MPAX experimental intermediate I_2 and the most folded is matched with MPAX experimental intermediate I_1 .

Although monomer unfolding simulations passed through structures with a good match to experimental intermediates I_2 and I_1 , no intermediates were populated (Fig. 6 A). As a result, this simulation did not perform as well in predicting intermediate structures I_2 and I_1 as the other simulation types. In good agreement with the MPAX experiments, all other simulations predicted two intermediates (two high probability peaks each for Fig. 6, B–D) except for the groups in which the peaks differ. Monomer refolding predicted groups C and D (Fig. 6 B), each of which shows an 80% match. Dimer unfolding predicted one intermediate peak on the cusp of groups A and B (85% match) and another in group C (65% match). Dimer refolding predicted groups D (60% match) and E (65% match).

On the whole, all simulations appeared to capture an order of folding events that is consistent with MPAX experiments. However, some simulation types perform better at populating the intermediate states that match best with the MPAX experimental intermediates. Clearly, monomer unfolding was the least successful, since it did not populate any intermediates. Using the sum of the two percentage matches between simulated and experimental intermediates in Table 2 as a score, the next best was dimer refolding ($60\% + 65\% = 135\%$), the second best was dimer unfolding ($85\% + 65\% = 150\%$), and the best predictor of the MPAX intermediates was monomer refolding ($80\% + 80\% = 160\%$).

The reasons for this order of the predictive success of γ TIM simulations—monomer_unfolding $<$ dimer_refolding $<$ dimer_unfolding $<$ monomer_refolding—is not entirely clear. One would think that the dimer simulations would be better than the monomer simulations, since this is a more accurate representation of the γ TIM protein in experiments. One possible reason why monomer refolding simulations provide a better match than dimer simulations is that the nature of the MPAX experiment and that of the simulations are not exactly the same. The MPAX experiment probes the equilibrium unfolding pathway of γ TIM under conditions favoring the native state with $\Delta G < 0$ (low denaturant). No kinetic simulation in this study exactly reproduces this experiment. Kinetic unfolding simulations study the unfolding pathway, but under conditions favoring the unfolded state (high temperature, native state, $\Delta G > 0$). On the other hand, kinetic refolding simulations are conducted under conditions favoring the folded state (low temperature, native state, $\Delta G < 0$), but initiate from an unfolded structure, which may lead to slight differences when comparing simulations and experiments.

All folding and unfolding kinetic γ TIM simulations predict a very similar folding order, as shown in Fig. 5, B–E. Therefore, it is likely that an equilibrium simulation, if feasible, would also produce a similar folding order. In previous studies using Gō models, the folding order has been shown to be highly conserved between kinetic simulations and equilibrium simulations (16,61). Therefore, the γ TIM folding order shown in Fig. 5, B–E, is likely to apply also to the folding of γ TIM in equilibrium simulations.

However, the probability of populating different values of Q (Fig. 6, A–D) does change significantly between unfolding and refolding simulations, as well as between monomer and dimer simulations. Although folding order appears to be robust between different simulation types (Fig. 5, B–E), the stability of the intermediate stages (i.e., “groups” in Fig. 6, A–D) is not. The successful prediction of monomer refolding over the dimer simulations may reflect a “lucky” shift in the stability of Q populations in monomer simulations to regions C and D, which match well with the MPAX experimental intermediates.

Regardless of these differences, nearly all the simulation types show remarkable success in predicting structures

TABLE 2 Match between the structures of high-population folding groups in simulations and MPAX experiments

Simulation	Group	MPAX intermediate	% match
Monomer unfolding	No intermediates		
Monomer refolding	C	2	80
	D	1	80
Dimer unfolding	A/B	2	85
	C	1	65
Dimer refolding	D	2	60
	E	1	65

The % match for all simulation groups are graphed in Fig. 9, A and B.

populated in the γ TIM folding/unfolding pathway, as determined by MPAX (34). Clearly, factoring in the effect of polypeptide chain entropy between short-range and long-range contacts through molecular dynamics simulations is extremely important in capturing the correct folding mechanism of γ TIM (67).

The generally good agreement between the folding pathway predicted by Gō-model MD simulations and that measured with MPAX experiments suggests that the dominant interactions are largely captured through a deterministic series of events (MD simulations), since stochastic events (used in Langevin simulations) were not explicitly included in the simulation. However, this agreement is not perfect, since the best match between the simulation and MPAX experiment is no greater than 90% (region B in Fig. 9 A). Furthermore, the intermediates populated do not consistently populate the regions that match best with experiments (Fig. 6, A, C, and D). Finally, experiments indicate that the two γ TIM monomers bind later (and unbind earlier) than the present MD simulations suggest (33,55,56). Including stochastic events through Langevin simulations may provide increased agreement with experimental results over the MD simulations.

Stochastic simulation events would randomly push the protein chain into conformations that a deterministic MD simulation might neglect. The random forces in Langevin simulations would likely alter the probability and structure of intermediate states populated during an MD simulation, although these differences are yet unknown. It may be found that Langevin simulations further improve the match with experiments compared to the simulations presented here. On the other hand, increasing the damping constant γ in Langevin dynamics may result in a worse agreement between simulations and experiments, which would argue against a significant role of stochastic events in guiding protein folding. In any case, the degree to which Langevin dynamics and molecular dynamics (from random initial conditions) differ for C_α Gō models has not been adequately explored. A high level of structural detail has been provided by solvent-exchange experiments, which have probed the folding pathway of a number of TIM barrels (34,36,40,42). These folding experiments provide a great opportunity to systematically explore the role of stochastic events in the

folding of a related family of proteins. Future investigations will use Langevin dynamics to investigate the impact of increasing stochastic events on the simulated folding of these TIM-barrel proteins.

CONCLUSIONS

Computational models of proteins offer many opportunities to study the vast amount of sequence and structural information available to the modern biochemist. Computer-based studies offer the possibility of rapidly identifying correlations and connections in vast data sets that are not often realized at the level of the bench biochemist. For computational approaches to be used effectively, the fundamental assumptions of the theoretical models employed must prove to be accurate and reliable at predicting real experimental results.

In predicting experimental protein folding pathways, the theoretical Gō model has demonstrated remarkable success (16,19–25). The Gō model is based on the assumption that the protein structure has been optimized such that the native structure is at the global energy minimum of all possible conformations in aqueous solution (26). In practice, the Gō model is tested by designing simulations in which all dihedrals and long-range contacts of the protein are set to have minimal energy when they match the x-ray crystal structure. This protein Gō model is then unfolded and refolded using molecular dynamics, and the pathway of unfolding and refolding in simulations is compared to experiments.

Gō models hold a great deal of promise as a key computational tool to investigate protein folding across different genomes. A structural class of proteins that is ripe for this genome-wide investigation is TIM barrels, predominantly metabolic proteins found in every organism (28). If the Gō model, or a modified version of the Gō model, is capable of accurate and repeated prediction of TIM-barrel folding pathways, an accurate automated folding analysis of TIM barrel structures in the PDB will be possible.

This study has performed such a comparison using a Gō model of γ TIM, and demonstrates that the match with experiments is very good (34). A previous study using the α -subunit of tryptophan synthase (α TS) also demonstrated excellent agreement between simulations and experiments (16). Thus far, the agreement appears excellent between Gō-model simulations, and experimental TIM-barrel folding looks very promising. However, more work needs to be done. The study of TIM barrels with residue-specific structural information (indole-3-glycerol phosphate synthase from *Sulfolobus solfataricus*, aldolase from rabbit, and TIM from humans) is currently underway. These studies will reveal whether the folding of other TIM barrels is equally well captured by the funneled energy landscape of the Gō model.

The fact that the γ TIM Gō model matches the γ TIM experiments supports the idea of an energy landscape of the

γ TIM protein that is “funneled” to the native state (2). Furthermore, it also indicates that the energy of each residue-residue contact is approximately equal throughout the protein chain. It is conceivable that other non-Gō energy parameters may also be successful in capturing the correct folding pathway of γ TIM (54,68). However, the fact that the Gō model does work well at predicting the folding pathways of γ TIM, α TS, and many other proteins testifies to a general applicability of this model (16,19–25).

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