

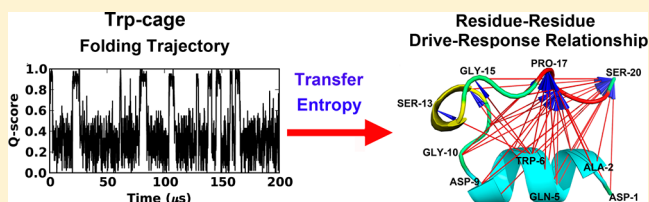
# Quantification of Drive-Response Relationships Between Residues During Protein Folding

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## S Supporting Information

**ABSTRACT:** Mutual correlation and cooperativity are commonly used to describe residue–residue interactions in protein folding/function. However, these metrics do not provide any information on the causality relationships between residues. Such drive-response relationships are poorly studied in protein folding/function and difficult to measure experimentally due to technical limitations. In this study, using the information theory transfer entropy (TE) that provides a direct measurement of causality between two times series, we have quantified the drive-response relationships between residues in the folding/unfolding processes of four small proteins generated by molecular dynamics simulations. Instead of using a time-averaged single TE value, the time-dependent TE is measured with the *Q*-scores based on residue–residue contacts and with the statistical significance analysis along the folding/unfolding processes. The TE analysis is able to identify the driving and responding residues that are different from the highly correlated residues revealed by the mutual information analysis. In general, the driving residues have more regular secondary structures, are more buried, and show greater effects on the protein stability as well as folding and unfolding rates. In addition, the dominant driving and responding residues from the TE analysis on the whole trajectory agree with those on a single folding event, demonstrating that the drive-response relationships are preserved in the nonequilibrium process. Our study provides detailed insights into the protein folding process and has potential applications in protein engineering and interpretation of time-dependent residue-based experimental observables for protein function.



## INTRODUCTION

Protein folding is one of the fundamental problems in biosciences. Different parts of a protein may have long-range interactions and fold in a concerted way. Such cooperative folding is considered to be essential to prevent the formation of partially unfolded structures that leads to aggregation.<sup>1,2</sup> In terms of specific residue–residue interactions, it is possible to measure their correlations experimentally using NMR spectroscopy.<sup>3–5</sup> Computational methods such as molecular dynamics (MD) simulations and anisotropic network modeling also provide valuable information about the intrinsic cooperativity in proteins' folding.<sup>6–8</sup> Pearson correlation and mutual information (MI) are two common measures to quantify the correlations. Specifically, MI incorporates both linear and nonlinear correlations and has been used in studying correlated motions of proteins.<sup>9–12</sup> However, one defect of these measures is that they are symmetric, and thus, it is not possible to distinguish one residue from another in a correlated pair. Knowing the directive interaction (i.e., the drive-response relationship) between residues is not only of theoretical interest but also can provide guidance in protein engineering.

A theoretic metric that can determine the drive-response relationships between residues is the information theory transfer entropy (TE) proposed by Schreiber.<sup>13</sup> TE quantifies the information flow from the past of one time series to the future of another time series. It has been used in finance<sup>14</sup> and

mostly in neuroscience to deduce the connection between neurons.<sup>15,16</sup> Recently, TE has been used in MD simulation trajectory analysis to elucidate the information flow in a transcription factor Ets-1.<sup>17</sup> Through the analysis of the simulation trajectories of the apo and holo states, the binding of DNA to H1 helix of Ets-1 appears to drive the correlated motion of the inhibitory helix HI-1 via a relay helix. The same method was applied to the autoactivation of extracellular signal-regulated kinases 1 and 2,<sup>18</sup> revealing how the helix-C at N-domain drives the fluctuation of the activation lip that may lead to activation. Another application of TE analysis is to identify important order parameters from MD simulations,<sup>19,20</sup> so that protein conformational changes can be better described by the change of these order parameters compared to those based on principle component analysis. These studies demonstrate that, when applied to MD simulation trajectory analysis, TE can be a valuable method in understanding the functional motions of proteins. However, the statistical significance of calculated TE values is not clearly addressed in these studies.<sup>17–20</sup> Since the calculated TE values are often very small, it is critical to use well-defined statistical significance analysis to warrant wide applications of the TE analysis in both computational and experimental studies.

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In this study, we apply the TE analysis to quantify the drive-response relationships between residues in the folding/unfolding processes of four small proteins generated by MD simulations. While it is well recognized that hydrophobic interactions are the driving force in protein folding,<sup>21</sup> to the best of our knowledge, to what extent each residue drives/responds to each other has not been studied before. In addition, the identified driving and responding residues are compared with those (correlated) residues identified by mutual information analysis; examined by the general properties such as secondary structure and solvent accessible surface area; and characterized by their effects on the protein stability as well as folding and unfolding rates. In terms of the computational and theoretical point of view, our study establishes statistical significance analysis of calculated TE values using simulation trajectories and determines if the stationary drive-response relationship is preserved in local, nonequilibrium events. Our understanding of protein function and underlying mechanisms of biologically important processes can be enriched by identifying residue-residue drive-response relationships through wide applications of TE analysis to MD simulations as well as time-dependent residue-based experimental observables.

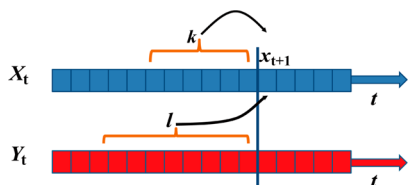
## MATERIALS AND METHODS

**Theory.** The all-atom folding trajectories were obtained from D. E. Shaw Research for their recent protein folding simulation study; the trajectories were saved every 200 ps and contained the coordinates of C $\alpha$  atoms.<sup>22</sup> Of the 12 proteins simulated, Trp-cage, BBL, Villin, and BBA were selected for this study because these proteins had multiple folding/unfolding events. To obtain a time series for each residue, we used a residue-based Q-score, that is, the fraction of native contacts formed for a specific residue. A native contact is counted when two C $\alpha$  atoms are closer than 1.2 times their distance in the native structure.

TE is a measure that quantifies the information flow from the past of one time series  $y(t)$  to the future of another time series  $x(t)$  (Figure 1). It was formally described by Schreiber<sup>13</sup> as

$$\text{TE}_{y \rightarrow x} = \sum p(x_{t+1}, x_t^{(k)}, y_t^{(l)}) \log \frac{p(x_{t+1}|x_t^{(k)}, y_t^{(l)})}{p(x_{t+1}|x_t^{(k)})} \quad (1)$$

where  $k$  and  $l$  are the embedding dimensions that are the number of steps to be included from the past,  $p()$  is the probability of one state,  $p(l)$  is the conditional probability, and the summation is over all possible combinations of states. By simple manipulation, TE can also be written in terms of



**Figure 1.** Schematic illustration of measuring TE.  $y(t)$  and  $x(t)$  are two time series. Let us assume that we try to predict the future of  $x(t)$ ,  $x_{t+1}$ , using the past  $k$  steps from  $x(t)$  and past  $l$  steps from  $y(t)$ . TE is the difference in such predictions between using the past of  $x(t)$  only and using the past of  $x(t)$  and  $y(t)$  together and quantifies the information flow from  $y(t)$  to  $x(t)$ .

Shannon entropy,  $H(x) = -\sum p(x_t) \log p(x_t)$ , which is the actual form used in the present calculations,

$$\begin{aligned} \text{TE}_{y \rightarrow x} &= H(x_{t+1}|x_t^{(k)}) - H(x_{t+1}|x_t^{(k)}, y_t^{(l)}) \\ &= H(x_{t+1}, x_t^{(k)}) + H(x_t^{(k)}, y_t^{(l)}) \\ &\quad - H(x_{t+1}, x_t^{(k)}, y_t^{(l)}) - H(x_t^{(k)}) \end{aligned} \quad (2)$$

where  $H(l)$  is conditional Shannon entropy. Due to finite sample size of the time series, two independent series can have (statistically insignificant) nonzero TE. To remove this bias, the shuffling method was used to calculate the effective TE ( $\text{TE}^{\text{eff}}$ ),<sup>14,17</sup> which is given by

$$\text{TE}_{y \rightarrow x}^{\text{eff}} = \text{TE}_{y \rightarrow x} - \frac{1}{N} \sum_{n=1}^N \text{TE}_{y_{\text{shuffled}} \rightarrow x} \quad (3)$$

where  $N$  is the number of shuffling and should be set to a sufficiently large number.<sup>23</sup> We used 500 for all calculations in this study. Using  $\text{TE}^{\text{eff}}$ , a normalized directional index can be derived as

$$D_{y \rightarrow x} = \frac{\text{TE}_{y \rightarrow x}^{\text{eff}}}{H(x_{t+1}|x_t^{(k)})} - \frac{\text{TE}_{x \rightarrow y}^{\text{eff}}}{H(y_{t+1}|y_t^{(l)})} \in [-1, 1] \quad (4)$$

where  $H(x_{t+1}|x_t^{(k)})$  and  $H(y_{t+1}|y_t^{(l)})$  are the maximal TE. A positive  $D$  value indicates information flow from  $y(t)$  to  $x(t)$  and vice versa for a negative value. For two completely independent time series,  $D_{y \rightarrow x}$  and  $\text{TE}^{\text{eff}}$  are 0. Our implementation of TE was verified with three numerical systems (Supporting Information (SI) section S1).

MI quantifies the difference of information between two time series (i.e., less correlation with more difference). For  $x(t)$  and  $y(t)$ , MI is

$$\text{MI}(x, y) = H(x) + H(y) - H(x, y) \quad (5)$$

We used a normalized MI as

$$\text{MI}'(x, y) = \sqrt{1 - e^{-2\text{MI}(x, y)}} \quad (6)$$

which ranges between 0 and 1.<sup>24</sup>

**Practical Computational Considerations.** From the practical computational point of view, several parameters affect the calculated TE values and must be determined carefully. The first one is symbolization, which is to map the time series to a finite set of symbols (i.e., discrete values or states) so that one can calculate the probability of having each symbol. Too many symbols result in poor estimation of probability density, while too few symbols usually fail to capture the dynamics of the system. One method of symbolization is to use the relative rank of each data point.<sup>25</sup> This method may reduce the state space under certain circumstances. A more intuitive method is to partition the data to several ranges and use the range index as symbol. As the residue Q-score has finite discrete states in nature, we directly used the number of native contacts formed for each residue as the symbol, which resulted in five symbols per residue on average.

The second crucial parameter is the embedding dimension  $k$  and  $l$  in eq 1 and Figure 1. The optimal dimension is system-dependent and difficult to be generalized. Although the false nearest-neighbors method has been used by others,<sup>17,26</sup> it does not guarantee the best solution and could converge to (unrealistic) high dimensions in the semistationary series studied here. High dimensions cause problems in probability

estimation as the state space is proportional to  $n^{k+l+1}$  in eq 2, where  $n$  is the average number of symbols per residue. With this in mind, we used low dimensions from 1 to 4 to examine if two window lengths 20 000 (4  $\mu$ s simulation time) and 50 000 (10  $\mu$ s) were sufficient to obtain meaningful time-dependent TE values. For each window size, we used the half of its length to slide over the whole trajectory. As TE can only be applied to stationary process, for each window along the simulation time, we used the augmented Dickey–Fuller method<sup>27</sup> to test its stationarity. The final results are based on embedding dimension 1 for both  $k$  and  $l$  because different dimension from 1 to 4 does not change the dominant driving and responding residues (data not shown). The time-dependent TE values were calculated with the window size of 10  $\mu$ s (for Trp-cage, BBL, and BBA) and 4  $\mu$ s (for Villin) as these window sizes showed 0% nonstationary windows along the simulation time. In the single folding (nonequilibrium) events, it was more difficult to use a window that was small enough to be stationary while large enough to contain statistically meaningful data points in this study. We finally used a window size of 2  $\mu$ s (i.e., 10 000 data points), which has 1.4% and 2.6% nonstationary windows for the two single folding events of BBL.

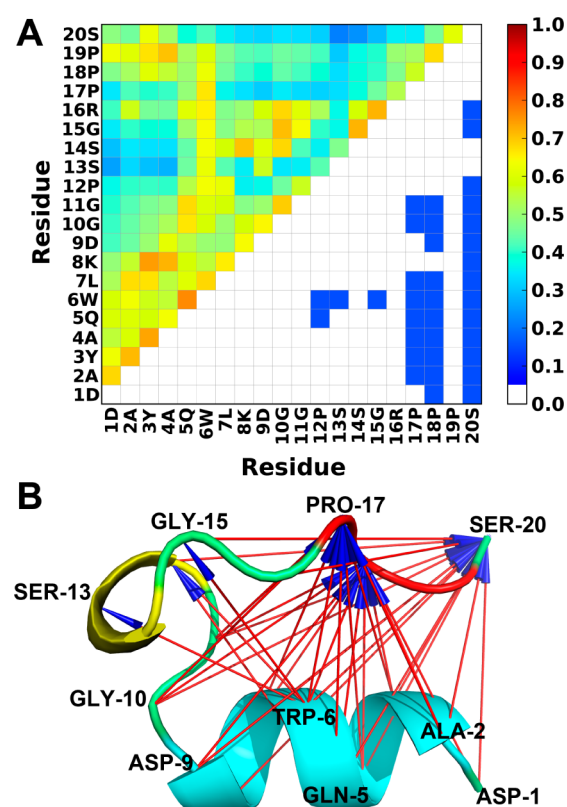
Finally, for each  $D_{y \rightarrow x}$  value in eq 4, it is highly desirable to assess its significance using statistical test. This is particularly important because  $D_{y \rightarrow x}$  is generally very small in magnitude (below 0.1), so that it is difficult to evaluate its statistical significance even with the shuffling method in eq 3. Therefore, we calculated the  $p$ -value using  $z$ -test against the random distributions of  $D_{y \rightarrow x}$  from shuffling. A cutoff of 0.1 was first applied to  $D_{y \rightarrow x}$  values to remove residue pairs that had small drive-response interactions, and then, a  $p$ -value cutoff of 0.05 was used to filter out a considerable number of pairs that were not significant (Table 1).

**Table 1. Number of Drive-Response Residue Pairs with and without the  $p$ -Value Cutoff of 0.05 for the Analysis of Whole Trajectories of the Test Proteins**

$p$ -value cutoff	Trp-cage	BBL	Villin	BBA
yes	36	17	16	8
no	82	149	133	42

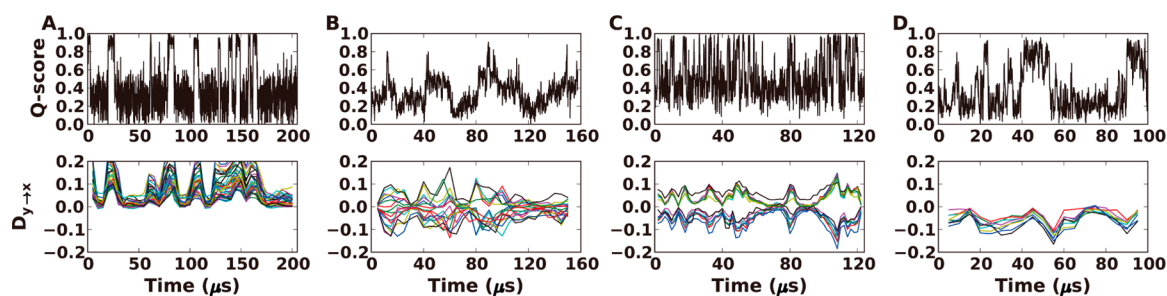
## RESULTS AND DISCUSSION

We applied the TE analysis to folding processes of four small proteins (Trp-cage, BBL, Villin, and BBA), whose trajectories were obtained from the recent protein folding simulations by Lindorff-Larsen et al. in D. E. Shaw Research,<sup>22</sup> and the simulation lengths were between 100 and 200  $\mu$ s. Briefly, a

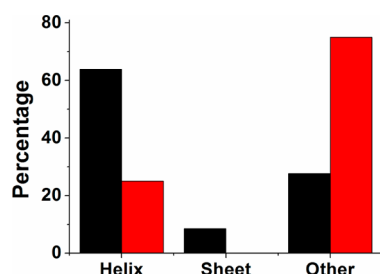


**Figure 3.** Driving and responding residues in Trp-cage folding/unfolding. (A) A driver-responder plot indicates a residue from  $x$ -axis drives (red) or responds to (blue) a residue from  $y$ -axis (lower-half triangle). Since the C-terminal residues respond to N-terminal residues in Trp-cage, no red color is shown. MI values, calculated from the whole trajectory, are colored based on the color-coding bar (upper-half triangle). MI values smaller than 0.05 are colored in white. (B) The structural view of the driving and responding residues. Arrows indicate driving interactions.  $\alpha$ -helix is in cyan,  $3_{10}$ -helix in yellow, and polyproline region in red.

residue Q-score was calculated for each residue along simulation time, and TE values were calculated between every residue pair in a time-dependent manner. The normalized directional index  $D_{y \rightarrow x}$  in eq 4, after a threshold cutoff and significance test (see Materials and Methods), indicates whether residue  $y$  drives ( $D_{y \rightarrow x} > 0$ ) or responds to ( $D_{y \rightarrow x} < 0$ ) residue  $x$ . The correspondence between the Q-score and  $D_{y \rightarrow x}$  values is remarkable (Figure 2), indicating that transfer of information entropy generally happens upon folding and unfolding events.



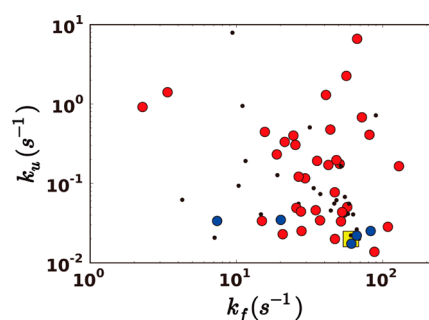
**Figure 2.** Time-dependent Q-score and  $D_{y \rightarrow x}$  profiles of the whole folding trajectory for (A) Trp-cage, (B) BBL, (C) Villin, and (D) BBA. Different colors in the  $D_{y \rightarrow x}$  profiles represent different residue pairs.



**Figure 4.** Percentages of different secondary structures of driving (black) and responding residues (red) from the test proteins.

To elucidate the drive-response relationships between residues, we calculated the time-average  $D_{y \rightarrow x}$  of each residue pair. Figure 3A (lower diagonal) shows the results of Trp-cage. Trp-cage is a small protein with only 20 amino acids and consists of an  $\alpha$ -helix, a  $3_{10}$ -helix, and a polyproline region at the C terminal (Figure 3B). Our results indicate that the folding of the polyproline region responds to the residues in the  $\alpha$ -helix. The rationalization of this behavior is clear with the Trp-cage structure. The N terminal helix forms first and then drives the formation of tertiary contacts of the polyproline region. It has been suggested that the folding of local structure followed by tertiary contact formation is a general mechanism of protein folding,<sup>22</sup> although the drive-response relationship can only be revealed by information analysis such as TE in this study. Notably, Trp-6, which is the most important residue in Trp-cage, is the dominant driver in our analysis, as it drives six residues. The interactions between Trp-6 and  $3_{10}$ -helix group as well as between Trp-6 and the polyproline region, which proved to be important in both experimental and computational studies,<sup>28,29</sup> are also captured in the TE analysis.

MI is a measure that quantifies the difference of information between two time series (i.e., less correlation with more difference). To compare TE with MI, we also calculated MI for each residue pair of Trp-cage (Figure 3A upper diagonal). The residue–residue correlations from MI are quite different from those from TE. In TE, the helical residues and the polyproline residues are correlated, while in MI, the correlation exists between N and C terminals, between residue 9–11 and 15–16, and between the helical residues. The differences between MI and TE also exist in BBL, BBA, and Villin (SI section S2). Such differences arise from the fact that our MI calculation only takes into account concurrent events. In other words, MI only calculates the correlations for the events that happen at the same time, while TE is able to incorporate the information from the past. A variation of MI, the time-delayed MI, which introduces a time lag to one of the series, is also able to capture causality in dynamic systems.<sup>30–32</sup> We applied time-delayed MI



**Figure 5.** Effects of mutations of driving and responding residues on protein L. Mutations of driving residues are in red circles, responding residues in blue circles, others in black dots, and the wild type values in yellow square.

to the test proteins, but it did not reveal any drive/response interactions (SI section S3).

Are there any general features of the driving and responding residues in folding and unfolding of proteins studied in this work? First, we check if a driving-responding relationship of a residue pair changes in time. A residue may drive another residue at one time and respond to the same residue at a later time. We counted the number of times  $D_{y \rightarrow x}$  changes its sign in the four proteins and found that none of the residue pairs changed their driving-responding relationships. This result not only justifies the use of time-average  $D_{y \rightarrow x}$  value to classify the drive-response relationship in Figure 3 but also suggests that residues play a constant role in the folding process. Second, we examine the relationship between the driving and responding residues and their locations in the context of protein structure. In Trp-cage, the driving residues are mostly in the buried helical region (Figure 3B). To examine whether this is a general feature, we calculated the secondary structures and relative solvent accessibility of each residue in the proteins. More than 70% of the driving residues are located in helix and sheet, but for the responding residues, the percentage of forming regular secondary structures is only 25% (Figure 4). For relative solvent accessibility to each residue, which is calculated as the percentage of the solvent accessible surface area (SASA) of a residue in the protein structure compared to that of a free residue, the driving residues have an average value of  $38.2 \pm 26.7\%$ , while the responding residues have an average of  $80.6 \pm 31.1\%$ . Thus, the driving residues are mostly buried at the hydrophobic core and have more regular secondary structures.

Is there any experimental support for the driving and responding residues identified by the TE analysis? Trp-6 in Trp-cage, which is the dominant driver, is already known to be of crucial importance to Trp-cage folding. For another protein, BBL, a small protein with downhill folding behavior under

**Table 2.** Effects of Mutations of Driving and Responding Residues on Experimental Denaturation Midpoint ( $T_m$ ), Folding ( $k_f$ ), and Unfolding Rates ( $k_u$ ) of BBL<sup>a</sup>

mutation to driving residues				mutation to responding residues			
mutation	$T_m$	$k_f$	$k_u$	mutation	$T_m$	$k_f$	$k_u$
V163A	−8.04	−72.0	1016.9	A148G	−1.7	−38.1	−24.7
T159S	−6.54	−60.3	181.8				
D162N	−12.3	−81.6	324.6				
I135A	−6.54	−50.6	459.7				

<sup>a</sup>The experimental data are shown as percentage changes compared to wild type values.  $T_m$ ,  $k_f$ , and  $k_u$  for wild type BBL are 327.3 K, 71 000 s<sup>−1</sup>, and 770 s<sup>−1</sup>.



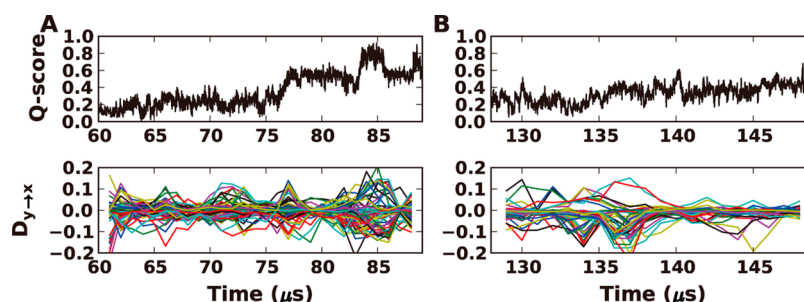


Figure 6. Q-score and  $D_{y \rightarrow x}$  profiles of two single folding events for BBL.

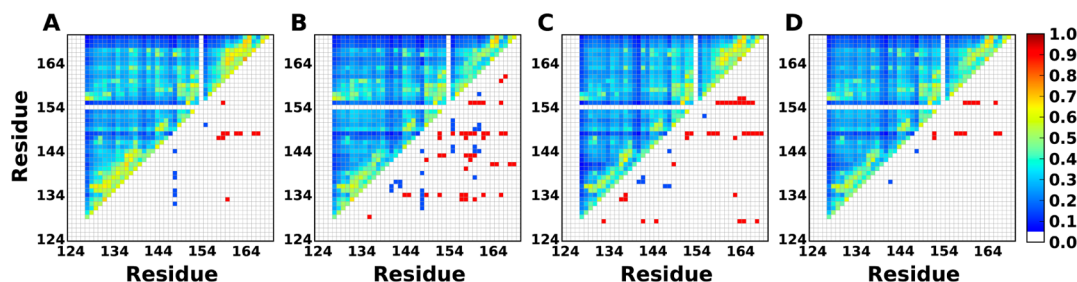


Figure 7. Driving and responding residues for BBL determined from (A) the whole trajectory and (B and C) two single folding events. (D) The shared drive-response residues and average MI from parts B and C. The plot schemes are the same as in Figure 3.

certain conditions, some experimental mutation data are available.<sup>33–35</sup> We compared the transition temperature ( $T_m$ ), the folding rate ( $k_f$ ), and the unfolding rate ( $k_u$ ) before and after the driving and responding residues are mutated (Table 2). Mutations to driving residues V163, T159, D162, and I135 decrease  $T_m$  and  $k_f$  by 6.54–12.3% and 50.6–81.6%, respectively, and increase  $k_u$  by 181.8–1016.9%. Mutation to the responding residue A148 has much smaller effects. No point-mutation experimental data are available for Villin and BBA, but we found almost every residue has been mutated for protein L, and  $k_u$  and  $k_f$  were also measured.<sup>36,37</sup> As protein L has 61 residues and the folding process is not easy to simulate with all-atom simulations, we used coarse-grained GO-type model<sup>38,39</sup> to generate folding/unfolding trajectories (SI section S4). Using the same analysis, we identified the driving and responding residues and compared how  $k_f$  and  $k_u$  changed after mutations (Figure 5). Similar to BBL, mutations to driving residues have greater effects on  $k_f$  and  $k_u$ .

So far, we focus on the whole folding trajectories with multiple folding events as a stationary process. To see if the drive-response relationships are preserved in single folding events (i.e., nonequilibrium process), we extracted two folding events from the trajectory of BBL (Figure 6): one from 60 to 90  $\mu$ s and the other from 125 to 150  $\mu$ s. It should be noted that the TE analysis can only be applied to a stationary process,<sup>13</sup> so it is necessary to use windowing method to partition the original single folding event to several stationary windows (see Materials and Methods). The  $D_{y \rightarrow x}$  profiles have correspondence with Q-score but are less evident. In terms of driving and responding residues, compared to the results from the whole trajectory (Figure 7A), the two single folding events are noisier and thus have more drive-response pairs (Figure 7B, C). However, when considering the pairs shared by the two single folding events, it is clear that the dominant responding residue A148 from the whole trajectory is still preserved (Figure 7D; red in lower-half triangle). Thus, the main characters of the

drive-response relationships from the whole trajectories are still captured in the single folding events.

## CONCLUDING REMARKS

In this study, we applied the TE analysis to the folding simulation trajectories of four small proteins, with the aim of identifying the drive-response relationship between residues and extending the TE analysis in a time-dependent manner with test of statistical significance. We find excellent correspondence between the folding process and the TE values between residues. Upon folding and unfolding, a large amount of information entropies is transferred between residues. Compared to responding residues, the driving residues are mostly buried at the hydrophobic core and form more regular secondary structures. They also have greater impacts on the protein stability as well as folding and unfolding rates. We also carried out the analysis on two single folding events from BBL and illustrate that the dominant responding residues from the whole trajectory are preserved in the single events.

The TE analysis can only be applied to a stationary process; that is, the average and standard deviation of the time series do not change over time. For a nonstationary process, using windowing method to cut the whole series into several segments could bypass this problem in principle. In analyzing the whole trajectories, we used two window sizes of 4 and 10  $\mu$ s and carried out the stationary test to make sure that each window was stationary. In the single folding events, it is more difficult to use a window that is small enough to fulfill the stationary requirement while large enough to contain sufficient data points. We ended up with a window size of 2  $\mu$ s, a compromise between the aforementioned two requirements, which resulted in 1.4% and 2.6% nonstationary windows for the two single folding events. The agreement of the dominant responding residues from the single folding events and the whole trajectories suggests that this small percentage of nonstationary window might be negligible. Another interesting observation is the time symmetric properties of the drive-

response relationship. In the whole folding trajectories, as the trajectories are time reversible, we do not expect any change between results calculated from the forward and backward time series. In the single folding events, even if residue A drives residue B in the forward (folding) process, residue B does not necessarily drive residue A in the backward (unfolding) process. The causality relationship of two residues depends not only on the sequential events, but also on the direction of information flow between them.

TE is applicable to any stationary time series in general. However, to get meaningful insights from the TE analysis, statistical significance and stationary tests should be performed carefully. The windowing method allows us to look at the causality in a time-dependent manner when time evolution of the system is of interest; it also provides an effective way to get around the stationary problem for nonstationary processes. As many processes in biology are nonequilibrium in nature and the experimental observables are thus mostly nonstationary, the wide applications of the TE analysis to such experimental observables are expected to reveal the underlying mechanisms of biologically important processes.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

S1: Transfer entropy in three numerical systems. S2: Driving and responding residues and MI for BBL, Villin, and BBA. S3: Time-delayed mutual information in three numerical systems and folding trajectories. S4: Coarse-grained simulation of protein L. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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