Temporally Overlapped but Uncoupled Motions in Dihydrofolate Reductase Catalysis

C. Tony Liu[†,‡], **Lin Wang**[†,‡], **Nina M. Goodey**[∥], **Philip Hanoian**[†], and **Stephen J. Benkovic**[*,†]

[†]Department of Chemistry, Pennsylvania State University, University Park, PA 16802, United States

[∥]Department of Chemistry and Biochemistry, Montclair State University, NJ 07043, United States

Abstract

Temporal correlations between protein motions and enzymatic reactions are often interpreted as evidence for catalytically important motions. Using dihydrofolate reductase as a model system, we show that there are many protein motions that temporally overlapped with the chemical reaction, and yet they do not exhibit the same kinetic behaviors (KIE and pH dependence) as the catalyzed chemical reaction. Thus, despite the temporal correlation, these motions are not directly coupled to the chemical transformation, and they might represent a different part of the catalytic cycle or simply be the product of the intrinsic flexibility of the protein.

The role of protein motions in enzyme catalysis has attracted great interest in contemporary enzymology. Protein motions ranging from picosecond to second have been implicated to be intimately involved in enzymatic processes, such as ligand binding, product dissociation, reorganization of the reactive Michaelis-Menten complex, and facilitating chemical reactions.[1–6] Perhaps the most intriguing observations are the millisecond to second timescale conformational changes that occur in conjunction with the chemical step, since these motions occur on the same timescale as most enzymatic reactions.[7] Thus, cases where millisecond timescale[8–13] conformational changes are observed concurrently with enzymatic reactions provide a tantalizing possibility for the direct 'coupling' (i.e., a causal and functional connection) between enzyme motions and catalysis. Furthermore, it has been demonstrated that mutations that hinder protein conformational changes can reduce the rates of enzymatic reactions,[2, 5, 14–17] further suggesting a link between motions and enzymatic reactions. Since the multidimensional free energy landscape of enzyme catalysis is characterized by an ensemble of inter-converting protein conformations,[2, 3, 18] many of the available protein motions may simply reflect the intrinsic thermal motions[5, 8, 19, 20] that exist in both the reactant and product states of the enzyme and do not directly facilitate the chemical reaction.

In order to gain further insight into the complex interplay between protein conformational changes and enzyme catalysis, we attached distance-sensitive fluorescent probe pairs onto *E. coli* dihydrofolate reductase (*ec*DHFR) and monitored the conformational fluctuations between various parts of the enzyme during the DHFR-catalyzed hydride transfer reaction

[*]**Corresponding Author**, sjb1@psu.edu. Phone: (814) 865-2882.
[‡]These authors contributed equally.

via the Förster resonance energy transfer (FRET) technique.[12] DHFR catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (H_2F) to yield 5,6,7,8-tetrahydrofolate (H_4F), which is involved in the biosynthesis of purines, thymidylate, and several amino acids.[21] The catalytic cycle of ecDHFR consists of five major complexes (Figure 1). X-ray crystallography[22] and NMR relaxation dispersion[9] data have shown significant conformational changes along the catalytic cycle, especially in the flexible active site Met20 loop (residues 9–24; Figure 1). Aside from the Met20 loop, conformational changes in other flexible regions of DHFR have not been extensively investigated, although many have been implicated as important for catalysis.[5, 9, 23, 24] Here, we probed the structural fluctuations in three flexible regions (Gly51, the GH loop, and the FG loop) and evaluated these data in light of previous results on the Met20 loop[12] to gain a more global insight into the motions involved during ecDHFR catalysis.

We utilized the same Alexa Fluor 555 maleimide – QSY 35 iodoacetamide FRET pair (abbreviated as AQ herein) system in a previous study to examine the conformational changes of the Met20 loop (Construct E; Table 2) during the chemical step.[12] The Förster distance (R_0) or the distance at which the energy transfer efficiency is at 50% for this pair is 24 Å.[12] Table 1 lists the residues that were labeled in the different FRET pair constructs. It should be noted that although constructs A–E were chosen to investigate the movements of flexible domains in the protein, the data here only probe the distance/geometry changes between the specific sites labeled. Increased fluorescence signals were detected in all cases when the E:NADPH:H_2F complex was converted to the E:NADP$^+$:H_4F complex, indicating enhanced FRET efficiency across the hydride transfer reaction. Structural data[22] of the model Michaelis-Menten complex vs. the E:NADP$^+$:H_4F product complex suggest that the enhanced FRET signals predominately arises from changes in the relative geometric orientations between the probes (Supporting Information).

Construct A was designed for examining the relationship between the adenosine binding subdomain and the major subdomain. It has been suggested that subdomain rotation during the catalytic cycle results in opening and closing of the cleft that houses the glutamate tail of dihydrofolate and tetrahydrofolate.[22] We found that at pH 7.0 the rate constants for the conformational change (measured by changes in the FRET intensity; k_{FRET}) and the enzymatic hydride transfer reaction (k_{hyd}) are essentially identical within experimental error (Table 2). Specifically, the conformational changes occurring during the conversion of E:NADPH:H_2F to E:NADP$^+$:H_4F cause a change in FRET signal (164±40 s^{-1}) at a rate similar to that obtained for the redox reaction (186±40 s^{-1}) between the substrate and cofactor. This observation suggests that these rates may correspond to the same kinetic event. If this is true, a perturbation affecting the activation energy barrier (or rate) of the chemical step should have a similar effect on the reaction-coupled motion. To test this hypothesis, we chose to perturb the rate of the chemical reaction using either isotopic substitution or changes in pH and to monitor the effects of these perturbations on k_{FRET}. Thus, these perturbations are expected to alter k_{FRET} only if the motions in question are indeed coupled to the rate of the hydride transfer reaction.

In wild-type ecDHFR, the hydride transfer rate is pH-dependent in the pH 6.0 – 8.5 range, and the k_{hyd} term exhibits a primary KIE of 3.[21] At pH 8.5 the hydride transfer rate in construct A was reduced to (58±5) s^{-1} whereas k_{FRET} remained unaffected (178±30 s^{-1}) in higher pH environments. Similarly, at pH 6.0 the k_{hyd} and k_{FRET} values were found to be (616±50) s^{-1} and (160±20) s^{-1}, respectively. Furthermore, no significant primary KIE was found for the k_{FRET} term at pH 7.0. This is similar to the Met20 loop movement studied previously using construct E,[12] where the fluorescence change associated with the FRET pair yielded a similar rate constant to that of the hydride transfer process at pH 7.0 but differed at pH 8.5 (k_{FRET} = 200 s^{-1}; k_{hyd} = 86 s^{-1}). Also, the k_{FRET} value in this system was

unaffected when NADPD was used as the cofactor rather than NADPH (KIE = 1.16±0.10). These data suggest that despite the temporal similarity, the conformational changes measure by k_{FRET} are distinct from the hydride transfer event.

When we examined the conformational changes around other flexible parts of the enzyme (constructs B, C, and E), we saw similar phenomena where rate constants of the fluorescence signal changes (k_{FRET}) associated with protein conformational fluctuations are nearly identical with the separately determined hydride transfer k_{hyd} rate constants at pH 7 (Table 2). However, none of the conformational changes probed in constructs A–E mirror the same kinetic behaviors as the catalyzed hydride transfer rate. The AQ FRET signal changes are either pH-independent (between pH 6.5–8.5) or insensitive to substrate isotope effect (near unity KIE observed with NADPD vs NADPH) or both, meaning that one can disambiguate the chemical step from the putatively coupled conformational changes by alteration of the experimental conditions. Thus, the protein motions observed here are not directly 'coupled' to the chemical step in such a way that an effect on one (either conformational motions or the catalyzed chemical reaction) directly translates to a similar kinetic outcome on the other. Additionally, it should be pointed out that in all constructs the turnover rate constants (k_{cat}) were found to be similar to the wild-type enzyme (Table S1). Thus, it is assumed that the rate limiting step in constructs A–E is still the release of the H_4F product from the E:NADPH:H_4F complex.[21]

We also examined the relationship between altered motions (i.e. via mutations) and the enzymatic reaction. G121 lies in the catalytically important FG loop of DHFR, and the rate reduction due to the G121V mutation has been interpreted as due to an alteration in the hydrogen bonding interactions between the FG loop and the Met20 loop that acts as a lid for the active site.[15, 25] However, a previous study showed that while the G121V mutation reduced the hydride transfer efficiency by ~100, fold the Met20 loop movement across the reaction coordinate was minimally disturbed (Table 2).[12] Similarly, we found the L54I mutation in construct A also lowered the hydride transfer rate by ~100 fold (Table 2) at pH 7.0. The L54I mutation also lowered the rate constant measured by the AQ FRET pair (k_{FRET} = 27±6 s^{-1} at pH 7.0), but only by about 6 times relative to the unmodified construct A. In both cases, the rate constants for the conformational changes (k_{FRET}) in the L54I variant of construct A and the G121V variant of construct E were pH independent between pH 6 – 8.5, illustrating that mutations that affect both conformational sampling and the chemical reaction do not necessarily entail a link between these two events. Also, we observed faster protein motions (k_{FRET}) than the catalyzed hydride transfer reaction (k_{hyd}) in the L54I variant of construct A and the G121V variant of construct E, indicating that the observed conformational changes are not rate-limited by the hydride transfer step or the slower turnover step in the catalytic cycle.

The catalytic impact of L54I might be comparable with a previous study which showed that systematic reduction in the size of the active site residue I14 (I14V, I14A, I14G) in *ec*DHFR dramatically affected the hydride transfer rate process.[26] In that particular study, decrease in the size of the hydrophobic residue, I14, in the active site resulted in larger average hydride donor-acceptor distances in the TS, which can be related to decreasing the hydride transfer rate constant. Structural data suggests direct van der Waals interaction between I14 and NADPH, perhaps contributing to a favorable orientation of NADPH in the TS for the reaction to occur. The same effect might be operative here, where the *p*-aminobenzoate group of the folate is in close van der Waals packing distance with L54 in the Michaelis-Menten complex (PDB: 7DFR).[22] Therefore, L54 might also be involved in proper substrate orientation.

Finally, we briefly examined the equilibrium thermally averaged C –C distance changes occurring during the hydride transfer reaction in wild-type *ec*DHFR using empirical valence bond molecular dynamics simulations (Supporting Information). For the residue pairs that were fluorescently labeled in constructs A to E we see either relatively subtle changes or no changes in the thermally averaged distance between the reactant state and the transition state (Table S3). This observation is consistent with the absence of an isotope or pH effect on the k_{FRET} component, as the k_{FRET} and k_{hyd} terms do not correspond to the same event. It is likely that the FRET data report on different protein movements than the ones captured by the simulations, but both the experimental and computational data agree that the motions followed here are not directly coupled to the chemical reaction.

In all seven constructs (Table 2) evaluated here, we demonstrated a disconnection between the measured conformational changes and the chemical transformation despite the initial temporal similarity between the two events. Protein motions that occur on the same timescale as the chemical reaction may simply reflect the interconversions between conformers within the same state instead of between different states (e.g. reactants to products). These motions might also be reflecting the intrinsic flexibility of the protein. Nonetheless, before we can advance our understanding of the relationship between conformational fluctuations and enzyme catalysis, more rigorous and direct evidence is necessary to clearly demonstrate that the motions of interest are indeed reporting on the chemical step. While small thermal equilibrium conformational fluctuations occurring as the system evolves from the RS to the TS of the hydride transfer reaction play an important role in reorganizing the reactive enzyme complex to facilitate the chemical transformation,[3, 5, 6] selective motions that directly coupled to chemical reactions remain experimentally elusive.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

ABBREVIATIONS

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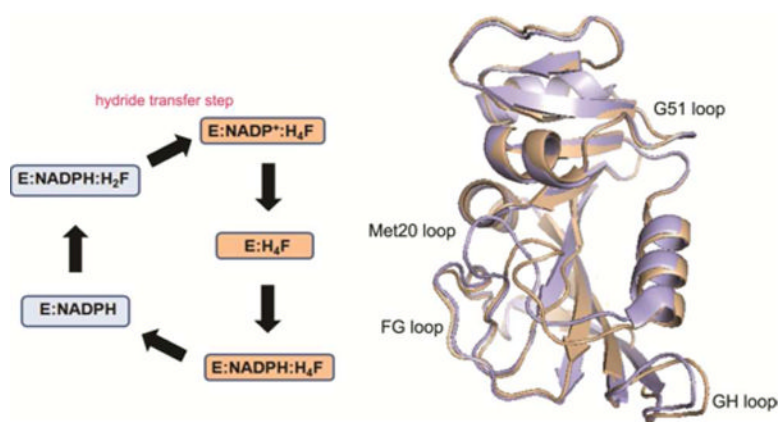
Figure 1.
Cartoon representations of the five major species[21] in the *ec*DHFR catalytic cycle on the left side and the superimposed crystal structures[22] of the modeled Michaelis-Menten complex (light purple; PDB 7DFR) and the initial product complex (bronze; PDB 1RC4) on the right side.
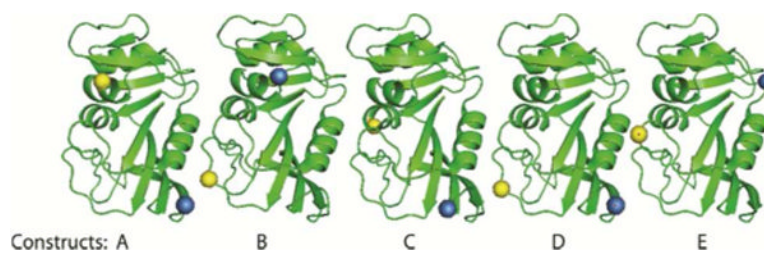
Figure 2.
Cartoon representation of *ec*DHFR constructs showing the locations (Table 1) where the FRET pairs were placed. Yellow spheres = QSY 35 Iodoacetamide labeling; Blue spheres = Alexa Fluor 555 maleimide labeling.

Table 1

Residue pairs with attached fluorescence probes.

Construct	Protein components	Residue pair
A	GH loop	48 – 145
B	G51 loop	51 – 120
C	GH loop	101 – 148
D	FG relative to GH loops	120 – 145
E[12]	Met20 loop	17 – 37

Table 2

Kinetic data associated with various *ec*DHFR constructs. ND = Not determined.

Construct	k_{hyd} at pH 17.0 (s^{-1})[3]	k_{FRET} at pH 17.0 (s^{-1})	k_{FRET} pH effect	k_{FRET} KIE
WT[1]	220 ± 10	NA	ND	ND
A	186 ± 40	164 ± 40	No	(0.9 ± 0.2)
A L54I	2.5 ± 1	27 ± 6	No	ND
B	172 ± 48	156 ± 42	No	ND
C	116 ± 30	122 ± 40	ND	(1.1 ± 0.1)
D	190 ± 20	2.0 ± 0.5	No	(1.1 ± 0.2)
E[2]	210 ± 40	282 ± 40	No	(1.2 ± 0.1)
E[2] G121V	3.5 ± 1	154 ± 40	ND	ND

[1] Data from reference (21);

[2] Data from reference (12);

[3] k_{hyd} values at different pH are provided in Table S1.