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Adaptations for Pressure and Temperature Effects on Loop Motion in *Escherichia coli* and *Moritella profunda* Dihydrofolate Reductase

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Abstract

Determining how enzymes in piezophilic microbes function at high pressure can give insights into how life adapts to living at high pressure. Here, the effects of pressure and temperature on loop motions are compared *Escherichia coli* (Ec) and *Moritella profunda* (Mp) dihydrofolate reductase (DHFR) via molecular dynamics simulations at combinations of the growth temperature and pressure of the two organisms. Analysis indicates that a flexible CD loop in MpDHFR is an adaptation for cold because it makes the adenosine binding subdomain more flexible. Also, analysis indicates that the Thr113-Glu27 hydrogen bond in MpDHFR is an adaptation for high pressure because it provides flexibility within the loop subdomain compared to the very strong Thr113-Asp27 hydrogen bond in EcDHFR, and affects the correlation of the Met20 and GH loops. In addition, the results suggest that temperature might affect external loops more strongly while pressure might affect motion between elements within the protein.

Graphical Abstract

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Declaration of interest.

The authors declare that they have no conflicts of interest.

Keywords

Piezophile; psychrophile; correlated motion

Introduction

Determining the effects of pressure and temperature on proteins, and the adaptations that Nature utilizes to protect against these effects, has implications for understanding how extremophiles adapt to thrive under extreme conditions [1, 2]. Adaptations for extremes, particularly of temperature, have often been studied by comparisons of homologous proteins from extremophilic (extreme-loving) and mesophilic (normal-loving) organisms. Since proteins must function at the growth temperature T_G and growth pressure P_G of the organism from which they came, the growth conditions can be considered to be “corresponding states” where proteins from extremophiles and mesophiles should have similar behavior [3, 4]. In particular, since both stability and flexibility are important for enzyme activity, homologous enzymes tend to have similar stability and flexibility near their corresponding states [5, 6]. However, since increasing stability also tends to increase rigidity while increasing flexibility tends to increase instability, stability at a particular extreme is not necessarily greater for an enzyme from an extremophile from that extreme if the need for flexibility is greater [5, 6]. In addition, adaptations for pressure have been less studied than adaptations for temperature. Moreover, while the effects of temperature on proteins can be ascribed to thermal motion,

the effects of pressure on proteins can vary significantly depending on the magnitude of the pressure and on the structure and function of the protein [7]. For instance, the role of pressure induced unfolding [8] in the stability-flexibility balance for proteins from piezophilic (pressure-loving) organisms is not clear.

Dihydrofolate reductase (DHFR), which catalyzes the reduction of dihydrofolate (DHF) by NADPH to tetrahydrofolate (THF), is ideal for comparative studies of extremophile enzymes because it is an essential enzyme for the biosynthesis of purine nucleotides and certain amino acids that has been found in organisms from all three kingdoms [9]. In addition, the structure-dynamics-function relationships of DHFR from *Escherichia coli* (Ec), which has a T_G of 37 °C at 0.1 MPa, have been highly studied [10, 11]. Extensive crystallographic studies have suggested that three conformations of the Met20 loop in EcDHFR (where the initial two letter code denotes the organism from which the DHFR was obtained) play an important role in the catalytic mechanism [12, 13]. Interestingly, NMR solution studies of the EcDHFR-folate complex have shown that while only the “occluded” conformation is observed at 3 MPa, the “open” conformation of the Met20 loop appears with increasing pressure, reaching almost 50% at 200 MPa [14]. Finally, analysis of several 10 ns MD simulations of wild-type and mutant EcDHFR suggest that mutations create changes in the correlation of motions in distant regions of the protein, which may be indicative of long-range interactions that influence function [15].

DHFR is also perhaps the most thoroughly studied enzyme from piezophiles. For instance, comparisons have been performed of the activity, stability, and structure of DHFR from *Moritella profunda* (Mp), which has a P_G of 22 MPa at 6 °C [16], with EcDHFR. MpDHFR is apparently adapted to living at high pressure since the activity of MpDHFR increases to 120% at 50 MPa relative to that at 0.1 MPa and then begins to decrease while wild-type EcDHFR shows monotonic inactivation to 47% at 250 MPa [17]. Interestingly, the relative activity of the Asp27Glu mutant of EcDHFR monotonically increases to 140% at 250 MPa accompanied by a change to a negative activation volume similar to MpDHFR and DHFRs from other deep-sea bacteria although the pressure-induced unfolding thermodynamics of the mutant remained similar to wild-type [18]. Since wild-type MpDHFR has a Glu27, this suggests that Glu27 may confer high pressure activity. However, whether this is due a difference in the catalytic role of Asp27 versus Glu27 is not clear. Moreover, MpDHFR is actually more sensitive to pressure denaturation since apo-MpDHFR has an unfolding pressure at 25 °C of only 70 MPa while apo-EcDHFR unfolds at 270 MPa [17]. In addition, the urea-induced unfolding thermodynamics of Asp27Glu EcDHFR remained similar to wild-type [18] while MpDHFR is more sensitive to thermal denaturation like other psychrophiles since apo-MpDHFR has an melting temperature at 0.1 MPa of 37.5 °C while apo-EcDHFR melts at ~13 °C higher [19]. Together this indicates that Glu27 is not contributing to differences in stability to thermal or pressure denaturation. In fact, whether MpDHFR is pressure-adapted or simply cold-adapted has been questioned [20].

Our group has examined the mean square fluctuations (MSF) of atomic coordinates in molecular dynamics (MD) simulations as a measure of the stability/flexibility balance needed for corresponding states behavior. Our simulations of MpDHFR and EcDHFR at 25 or 37 °C and 0.1 or 22 MPa [21] indicate that MSF on a timescale of ~10 ns, averaged over

the heavy atoms, are a potential marker for corresponding states behavior because they become similar at T_G and P_G of the organism from which the DHFR was isolated. The simulations also indicate that ns-plus MSF increase with both temperature and pressure, while sub-ns MSF increase with temperature but decrease slightly with pressure. In other work, our quasiharmonic approximation for energy landscapes (QHAEL) analysis [22] of EcDHFR and MpDHFR at different temperatures and pressures [23] indicate that temperature widens the underlying local potential wells while pressure makes them steeper, which is consistent with the previous observations. Also, the increased ns-plus timescale MSF at higher pressure appear to be associated with collective motions because they appear above the glass transition. Finally, the analysis shows that the underlying local potential wells of MpDHFR are softer than those of EcDHFR.

Here, the sequence differences that lead to adaptations to pressure and temperature are examined in EcDHFR and MpDHFR by analyzing molecular dynamics simulations at various temperatures and pressures. The effects of pressure and temperature on the loop motions, particularly the Met20 loop, are compared. The differences in MSF of C_α atoms are considered in light of changes in the correlations of motion via covariances of the MSF. Then, sequence differences between EcDHFR and MpDHFR that lead to differences in either hydrogen bond occupancies and lifetimes are identified, since highly occupied hydrogen bonds are likely to reduce the MSF while longer lifetimes are indicative of stronger hydrogen bonds. These hydrogen bonds are examined in light of differences in loop motion identified in the MSF. These results plus experimental data suggest that a flexible CD loop may be an adaptation for cold temperatures in MpDHFR while a flexible hydrogen bond between Thr113 and Glu27 may be an adaptation for pressure.

Methods

The simulations analyzed here have been previously reported [21], so the methods are described briefly here and additional details can be found in the previous work. The starting coordinates and sequences for the proteins in the previous work were obtained from the Protein Data Bank (PDB) [24] for EcDHFR (PDB ID: 1RX2) [12] and MpDHFR (PDB ID: 2ZZA, which lacks coordinates for Arg162) [25] with THF built in. Sequence alignment of EcDHFR and MpDHFR was performed using Clustal Omega [26] except for residues 66 to 69 of EcDHFR. MD simulations and other coordinate manipulations were performed using the molecular mechanics package CHARMM version 37b2 and version 40b2 [27]. The set-up was performed in CHARMMing [28] using default protocols except as noted. The CHARMM36 all-atom nonpolarizable potential energy parameter set [29, 30] was used for the proteins. However, water was modeled by TIP4P-Ew [31] because of the importance of the properties of water under pressure. Additionally, a force field for THF was generated using the CHARMM Generalized Force Field server (cgenff.paramchem.org). The simulations utilized the leapfrog Verlet algorithm with a time step of 0.001 ps and were maintained in the NPT ensemble with the Nosé-Hoover algorithm [32, 33] for the thermostat and barostat. Periodic boundary conditions and the particle mesh Ewald (PME) summation algorithm with a k -space grid spacing of about 0.34 Å [34] were used. The rhombic dodecahedral simulation box has a distance between faces of ~71 Å and each protein was in 0.15 M KCl, so that there are 7414 water molecules, 26 K^+ , and 15 Cl^- for MpDHFR and

7420 water molecules 27 K^+ and 14 Cl^- for EcDHFR. After heating for 100 ps and pressurizing the 22 MPa simulations for 22 ps, the simulations were allowed to equilibrate unperturbed for 4 ns after which the 50 ns production run was collected.

Average properties were calculated from coordinates written at a 1 ps except as noted. Hydrogen bonds were defined as having a distance between the donor hydrogen i and acceptor atom j smaller than 2.40 Å and the angle of D-H...A larger than 130°. Lifetimes were calculated in the CHARMM analysis facility. Two hydrogen bonds simultaneously formed with the same protein atom were calculated as two separate events. The 10 ns MSF of protein heavy atoms were calculated with respect to the average structure of 10 ns blocks. The statistics for the 10 ns MSF of protein heavy atoms and number of hydrogen bonds were obtained from block averaging the 10 ns blocks. The MSF of C_α atoms and covariance maps of these fluctuations were calculated from the entire 50 ns from the average structure over the 50 ns.

Results

General Effects of Temperature and Pressure

Analyses of the simulations of EcDHFR and MpDHFR at different temperatures and pressures are presented using a consensus sequence based on the numbering in EcDHFR (Fig. 1). Specifically, residues Val1 and Asp68 of MpDHFR (using MpDHFR numbering) are referred as Val0 and Asp66.5 here. The loop regions are highlighted in both the sequences (Fig. 1) and crystal structures (Fig. 2). EcDHFR has two subdomains, which each move as rigid bodies [12]. The loop subdomain contains the Met20, FG, and GH loops, the strands A, F, G, and H of the β -sheet, and helix B while the adenosine binding subdomain contains the CD loop, the strands B, C, D, and E of the β -sheet, and the helices C, E, and F.

To give a general overview of the characteristics of EcDHFR and MpDHFR in the simulations, the average MSF of the protein heavy atoms and the average number of hydrogen bonds N_{HB} are given in Table 1. Generally, the magnitude of the MSF varies inversely with N_{HB} and the average MSF for the two DHFR are similar at the T_G - P_G of the organism that the DHFR is from. The simulations of MpDHFR at 310 K at both pressures appear barely stable, which is consistent with the experimental melting temperature of MpDHFR:DHF at 0.1 MPa of 316.15 ± 0.3 K [19], so the fluctuations may be only lower estimates; however, large fluctuations indicate the most temperature sensitive regions. In addition, increasing the temperature at constant pressure results in one fewer hydrogen bond in EcDHFR but two to three fewer in MpDHFR while increasing the pressure at constant temperature results in two fewer hydrogen bonds in EcDHFR but about the same number of bonds in MpDHFR. This suggests that MpDHFR might be more sensitive to higher temperatures while EcDHFR might be more sensitive to higher pressures. In addition, at a given temperature and pressure, EcDHFR has one to five more hydrogen bonds than MpDHFR, indicating that EcDHFR has more interactions, which could be responsible for its smaller MSF.

Localizing and Characterizing Mobile Regions.

The MSF of the C_α atoms as a function of residue number for EcDHFR and MpDHFR were calculated to localize regions where high temperature or high pressure may be disrupting the protein, and are shown at a common state of 279 K, 0.1 MPa, at a raised temperature state of 310 K, 0.1 MPa (T_G - P_G of EcDHFR), and at a raised pressure state of 279 K, 22 MPa (T_G - P_G of MpDHFR) (Fig. 3); results at 310 K, 22 MPa are in the Supplementary Materials (Fig. S1). Overall, the MSF for EcDHFR and MpDHFR look most similar at the T_G - P_G for parent organism from which the DHFR came; i.e., for EcDHFR at 310 K, 0.1 MPa (green line in Fig. 3a) and MpDHFR at 279 K, 22 MPa (blue line in Fig. 3b). The MSF generally increase over the entire protein for both EcDHFR and MpDHFR as the temperature increases from 279 to 310 K (black versus green lines in Fig. 3), although MpDHFR shows much greater sensitivity to high temperature, with large increases in the MSF in the Met20, CD, and GH loops. In addition, the MSF generally increase almost exclusively in the loop regions as pressure increases from 1 to 22 MPa (black versus blue lines in Fig. 3), although EcDHFR shows much greater sensitivity to high pressure with large increases of the MSF in the GH and Met20 loops, while MpDHFR shows a small increase in the CD loop.

The covariance matrices of the C_α atomic fluctuations for EcDHFR and MpDHFR are also shown at 279 K, 0.1 MPa, at 310 K, 0.1 MPa, and at 279 K, 22 MPa (Fig. 4); results at 310 K, 22 MPa as well as inter-residue distance matrices are in the Supplementary Materials (Fig. S2, S3). Overall, the covariance matrices for EcDHFR and MpDHFR also look most similar at the T_G - P_G for parent organism from which the DHFR came; i.e., for EcDHFR at 310 K, 0.1 MPa (uppermost, Fig. 4a) and MpDHFR at 279 K, 22 MPa (lowermost, Fig. 4b). Positive correlations between two regions indicate that they are moving together while negative correlations indicate that they are moving in opposition as separate groups. Of note is the positive correlation of the Met20 and GH loops, which increases from that at the common state (middle, Fig. 4ab) when the temperature is increased to 310 K for EcDHFR and when the pressure is increased to 22 MPa for MpDHFR. The negative correlations increase for the GH loop of EcDHFR more when the pressure is increased than when the temperature is increased while they greatly increase for the all of the loops of MpDHFR when the temperature is increased but increase for some residues but decrease for others when the pressure is increased.

Sequence Differences and Temperature Sensitivity at Constant Pressure.

Since MpDHFR is thermally less stable than EcDHFR (its T_m is lower by $\sim 13^\circ\text{C}$ for the apo-enzyme [19]), possible origins of the thermal instability are examined first by comparing the 279 K, 0.1 MPa and 310 K, 0.1 MPa covariances (middle and top, respectively, in Fig. 4) and hydrogen bonds. A caveat for the implications of the findings here for thermal or pressure stability is that only the folded native states of the protein are examined in the simulations and not the unfolded states. However, the sequence differences described here change the hydrogen bonds and so are pointed out only as possible origins of thermal instability.

The large MSF of the CD loop in MpDHFR at 310 K (Fig. 3b) appears to be due to its anticorrelated motion with the rest of the adenosine binding subdomain rather than the rigid

body motion of the entire adenosine binding subdomain in EcDHFR, which is the most dramatic difference with EcDHFR in the covariance matrices (Fig. 4). The CD loop in EcDHFR appears to be relatively rigid at 310 K because of a hydrogen bond between Arg71NH and Asp69ODx, which remains mostly occupied even at the higher temperature ($\tau = 230$ ps, occupancy of 0.77 at 279 K vs. $\tau = 26$ ps, occupancy of 0.52 at 310 K). In contrast, the CD loop of MpDHFR is much more flexible because there is no such hydrogen bond since a glycine and a proline, respectively, are at the equivalent positions (Fig. 2) as well as an extra residue Asp66.5 (Fig. 1), giving rise to the large mobility at 310 K.

The large MSF of the Met20 and GH loops in MpDHFR at 310 K (Fig. 3b) appear to be due to weaker hydrogen bonds in the loop subdomain. In EcDHFR, a very strong hydrogen bond between Thr113OG1 in strand βF and Asp27OD1 in helix B, which remains fully occupied with a very long lifetime at 310 K ($\tau = \sim 25$ ns, occupancy of 1 at 279 K vs. $\tau = \sim 6$ ns, occupancy of 1 at 310 K), restricts the movement of helix B relative to the β -sheet. This restricts the motion of the Met20 loop, which connects strand βA (adjacent to βF) with helix B. In addition, the restricted motion of helix B stabilizes a hydrogen bond between Ala26N of helix B and Asn147OD of the GH loop ($\tau = 5$ ps, occupancy of 0.74 at 279 K vs. $\tau = 5$ ps, occupancy of 0.61 at 310 K). Thus, the Met20 and GH loops are correlated in EcDHFR at 310 K (top, Fig. 4a). On the other hand, the hydrogen bond between Thr113OG1 and Glu27OE2 in MpDHFR is much weaker than the equivalent one in EcDHFR and is only somewhat occupied at the higher temperature ($\tau = 87$ ps, occupancy of 0.30 at 279 K vs. $\tau = 37$ ps, occupancy of 0.10 at 310 K), which allows helix B to move relative to the β -sheet. The helix B motion causes the Met20 loop to move more (Fig. 2) and also breaks the hydrogen bond between Ala26N in helix B and Asn147OD in the GH loop, which is essentially unoccupied at 310 K ($\tau = 11$ ps, occupancy of 0.62 at 279 K vs. $\tau = 6$ ps, occupancy of 0.04 at 310 K). This means the GH loop can move independently of the Met20 loop in MpDHFR at 310 K (top, Fig. 4b) so both move too much.

Sequence Differences and Pressure Sensitivity at Constant Temperature.

Since EcDHFR has reduced activity with increasing pressure ($\sim 88\%$ relative activity at 25 MPa [17]), possible origins of the pressure inactivation are examined next by comparing the 279 K, 0.1 MPa with 279 K, 22 MPa covariances (middle and bottom, respectively, in Fig. 4) and hydrogen bonds.

The hydrogen bond of Thr113 with Asp27 in EcDHFR mentioned above does not appear to be affected by higher pressure ($\tau = 25$ ns, occupancy of 1 at 0.1 MPa vs. $\tau = 25$ ns, occupancy of 1 at 22 MPa). However, the Ala26N-Asn147OD hydrogen bond between helix B and GH loop weakens considerably in EcDHFR at the higher pressure ($\tau = 5$ ps, occupancy of 0.74 at 0.1 MPa vs. $\tau = 9$ ps, occupancy of 0.20 at 22 MPa) much like the same hydrogen bond in MpDHFR at the higher temperature, so the Met20 loop is independent of the GH loop of EcDHFR at 22 MPa (bottom, Fig. 4a), so both move too much like MpDHFR at 310 K (top, Fig. 4b). On the other hand, the Thr113 to Glu27 hydrogen bond in MpDHFR becomes more occupied at the higher pressure ($\tau = 87$ ps, occupancy of 0.30 at 0.1 MPa vs. $\tau = 48$ ps, occupancy of 0.76 at 22 MPa). Interestingly, the Ala26N-Asn147OD hydrogen bond becomes somewhat more occupied with increasing

pressure ($\tau = 11$ ps, occupancy of 0.62 at 0.1 MPa vs. $\tau = 11$ ps, occupancy of 0.88 at 22 MPa), so helix B and GH loop remain correlated. This means the Met20 and GH loops move in a slightly more correlated manner in MpDHFR at 22 MPa (bottom, Fig. 4b), much like that in EcDHFR at 310 K (top, Fig. 4a). In other words, they move more similarly at their growth conditions. In addition, this suggests that the greater flexibility of the Thr113-Glu27 hydrogen bond may allow sufficient motion to preserve the Ala26N-Asn147OD hydrogen bond.

Another mutation in MpDHFR may further stabilize the GH loop at high pressures. The hydrogen bond of Ala143N with Asn150OD1 in MpDHFR is highly occupied regardless of pressure ($\tau = 11$ ps, occupancy of 0.87 at 0.1 MPa vs. $\tau = 14$ ps, occupancy of 0.90 at 22 MPa) compared to Ala143N with Ser150OG in EcDHFR, which is relatively unoccupied ($\tau = 2$ ps, occupancy of 0.17 at 0.1 MPa vs. $\tau = 2$ ps, occupancy of 0.10 at 22 MPa). In the simulation, the movement of the GH loop in MpDHFR at 22 MPa was observed to be restricted by this hydrogen bond.

Finally, the CD loop does not seem to be sensitive to high pressure, at least at 22 MPa. The Arg71NH-Asp69ODx hydrogen bond in EcDHFR appears to remain occupied at the higher pressure ($\tau = 230$ ps, occupancy of 0.77 at 0.1 MPa vs. $\tau = 167$ ps, occupancy of 0.63 at 22 MPa). In addition, the lack of this hydrogen bond and the extra residue in MpDHFR do not seem to affect the MSF (Fig. 3).

Discussion

Overall, the MSF and the covariances of the MSF for EcDHFR and MpDHFR look most similar at the T_G - P_G for parent organism from which the DHFR came, illustrating that T_G - P_G appear to be the corresponding states as indicated previously [21]. This appears to be accomplished by sequence differences in hydrogen bonds between the two.

EcDHFR has been experimentally observed to be more thermally stable than MpDHFR [19]. Although the unfolded state was not examined here as mentioned above, the simulations indicate two possible contributions – the more rigid CD loop and the stronger 113-27 hydrogen bond – to the phenomena, but only the first is supported by experiment. A more rigid CD loop appears to stabilize the adenosine binding subdomain, which moves as a rigid body [12]. The simulations show that the CD loop in EcDHFR is more rigid due to a hydrogen bond between Arg71 and Asp69 while the same loop in MpDHFR is more flexible because an equivalent hydrogen bond cannot form between Gly71 and Pro69 and it has an additional residue. Thus, a more flexible CD loop appears to be an adaptation for flexibility at low temperatures. Sequence data also supports this since Gly71, a non-hydrogen bonding residue at 69, and an extra loop residue appear in DHFR from other deep sea-bacteria as well as some surface bacteria of the same genus that have low free energies of urea unfolding similar to the deep-sea bacteria [35, 36]. The other possible contribution is that a strong 113-27 hydrogen bond could potentially stabilize the loop subdomain, which also moves as a rigid body [12], by connecting the lower half of the β -sheet with helix B. The simulations indicate that the very strong hydrogen bond between Thr113 and Asp27 in EcDHFR persists at the higher temperature while the weaker one between Thr113 and

Glu27 in MpDHFR is broken. However, since the site-specific mutation Asp27Glu in EcDHFR does not lead to thermal instability experimentally [18], the breaking of the Thr113-Glu27 hydrogen bond in MpDHFR at the higher temperature is more likely a consequence of the overall greater flexibility of MpDHFR than a cause. Specifically, since the adenosine binding domain lies between the two halves of the sequence of the loop domain, a more flexible adenosine binding domain would also make a more flexible loop domain. Thus, the difference between an Asp27 or a Glu27 does not appear to affect stability.

MpDHFR has been experimentally observed to retain atmospheric pressure activity to higher pressures than EcDHFR [17]. The simulations indicate two possible contributions – the weaker 113-27 hydrogen bond and the stronger 143-150 hydrogen bond. For the first, the simulations suggest that the weaker Thr113-Glu27 hydrogen bond decreases the correlation between the Met20 and GH loops in MpDHFR while the strong Thr113-Asp27 hydrogen bond leads to strong correlation in EcDHFR. Since pressure will decrease the available volume, this suggests that the flexible loop subdomain allows helix B to remain connected to the GH loop. The importance of residue 27 is supported by the experimental observation that Asp27Glu EcDHFR has continuously increasing activity with increasing pressure [18], although sequences from deep-sea *Shewanella* indicate that it is not the only adaptation that can lead to pressure insensitivity [36]. In addition, the appearance of open Met20 conformation [14] and the decrease in activity [17] in EcDHFR at 200 MPa further suggests the loss of activity is associated with a conformational change. The other possible contribution is that the Ala143-Asn150 hydrogen bond stabilizes the GH loop in MpDHFR compared to the Ala143-Ser150 in EcDHFR. However, since Asp27Glu EcDHFR works well at high pressure without the additional mutation of Ser150Asn, the latter may help protect the relatively fragile MpDHFR at higher pressures but is probably not necessary unless the DHFR is fragile. Interestingly, sequences from deep-sea *Shewanella* indicate they might have more rigid GH loops as well since they have a Glu150, which can presumably form a strong hydrogen bond [36]. Finally, since Asp27Glu EcDHFR works well at high pressure without the additional mutations of Arg71Gly and Asp69Pro, this further supports that a flexible CD loop may be an adaptation for cold temperatures rather than high pressure and may contribute to the low unfolding pressure in MpDHFR. Since no studies have been reported on the effects of site-specific mutations of MpDHFR, temperature and pressure dependent studies are warranted.

Overall, these results suggest that the identity of residue 27 affects pressure sensitivity of the activity of DHFR because it affects the correlation of the Met20 and GH loops, or at least the motion of Met20, rather than a change in catalytic activity. Thus, the observed increase in activity of MpDHFR up to 50 MPa [17] might be due to the more flexible hydrogen bond between Thr113 and Glu27 while the observed decrease in activity of MpDHFR with pressure above 50 MPa [17] might be due to initial pressure unfolding, which occurs in the apo-enzyme at 70 MPa [17]. In addition, since thermal instability is a characteristic of enzymes from psychrophiles so that they can be flexible enough at low temperatures [5, 6], the decrease in activity is most likely due to adaptations for low temperatures. On the other hand, the Glu27 adaptation indicates that adaptations that increase internal flexibility could prevent pressure inactivation because pressure tends to decrease available space for relative

motion inside the protein. Thus, since the cold adaptation here makes an exterior loop more flexible while the pressure adaptation makes an interior connection between two secondary structural elements more flexible, this suggests that different adaptations for flexibility are necessary for cold temperatures versus high pressures.

As a final note, the results also suggest further experimental mutational studies. First, although the Asp27Glu mutation of EcDHFR has been made, the Glu27Asp mutation of MpDHFR has not been reported to our knowledge. The results here suggest that in MpDHFR, either the double mutation of Glu71Arg and Pro69Asp to stabilize the adenosine binding domain or an Asn150Ser mutation to give extra flexibility of the GH loop in the loop domain might be necessary to make Glu27Asp MpDHFR active. Conversely, the double mutation of Arg71Gly and Asp69Pro in either wild-type or Asp27 Glu EcDHFR might make it more active at low temperatures.

Conclusions

Overall, the results here indicate that a more flexible CD loop in MpDHFR is an adaptation for low temperature while a more flexible interaction between two secondary structure elements is an adaptation for high pressure. The more flexible CD loop in MpDHFR due to one less hydrogen bond and one more residue appears to make the protein more flexible especially in the adenosine binding subdomain. In addition, the more flexible interaction between two secondary structure elements due to the weaker hydrogen bond in MpDHFR appears to allow the GH loop to become more correlated with the Met20 loop at the T_G - P_G of MpDHFR, which is a characteristic of EcDHFR at its T_G - P_G .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 2.

The Met20 (orange), CD (cyan), FG (light green), and GH (magenta) loops are shown in crystal structures of a) EcDHFR and b) MpDHFR. The adenosine binding domain contains the top half of the β -sheet, the upper three α -helices, and the topmost loop while the loop domain contains the lower half of the β -sheet, the α -helix on the lower right, and the lower three loops. Important hydrogen bonds are also highlighted.

Figure 3. MSF of Ca atoms in a) EcDHFR and b) MpDHFR at 279 K, 0.1 MPa (black); 310 K, 0.1 MPa (green); 279 K, 22 MPa (blue). The Met20 (orange), CD (cyan), FG (light green), and GH (magenta) loops are highlighted. Hydrogen bonds occurring between residues 113 and 27 are denoted by '*' and between 150 and 143 are denoted by 'x'.

Figure 4. Covariance matrices of C_{α} atomic fluctuations in a) EcDHFR and b) MpDHFR. The Met20 (orange), CD (cyan), FG (light green), and GH (magenta) loops are highlighted on the axes. Hydrogen bonds are plotted as “x” at the donor residue y and the acceptor residue x with the lifetime indicated by the size. EcDHFR Asp27 OD1 is not shown to scale because it has a lifetime of ~25 ns at 279 K, which is the largest among all.

Table 1.

10 ns mean-square fluctuations of heavy atoms (MSF) and number of hydrogen bonds (N_{HB}) in EcDHFR and MpDHFR at different temperatures and pressures.

T, P (K, MPa)	$\text{MSF} (\text{\AA}^2)$		N_{HB}	
	EcDHFR	MpDHFR	EcDHFR	MpDHFR
279, 0.1	0.65±0.02	0.76±0.08	107±2	103±2
279, 22	0.71±0.11	0.83±0.04	105±1	104±1
310, 0.1	0.93±0.07	1.39±0.15	106±1	101±2
310, 22	1.04±0.14	1.49±0.16	104±1	101±2