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Identification of endogenous ligands bound to bacterially expressed human and *E. coli* dihydrofolate reductase by 2D NMR

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Abstract

Dihydrofolate reductase (DHFR) is a well-studied drug target and a paradigm for understanding enzyme catalysis. Preparation of pure DHFR samples, in defined ligand-bound states, is a prerequisite for *in vitro* studies and drug discovery efforts. We use NMR spectroscopy to monitor ligand content of human and *E. coli* DHFR (ecDHFR), which bind different co-purifying ligands during expression in bacteria. An alternate purification strategy yields highly pure DHFR complexes, containing only the desired ligands, in the quantities required for structural studies. Interestingly, ecDHFR is bound to endogenous THF while human DHFR is bound to NADP. Consistent with these findings, a designed “humanized” mutant of ecDHFR switches binding specificity in the cell.

Introduction

Dihydrofolate reductase (DHFR) reduces dihydrofolate (DHF) to tetrahydrofolate (THF), and is most often the sole source of THF in cells. THF is a precursor for thymidine synthesis, and therefore for DNA replication and cell proliferation. Due to its critical cellular function, DHFR has been studied intensively for many years, as a paradigm for understanding enzyme catalysis and as a target for anticancer and antibiotic drugs [1,2,3,4,5,6]. Selection of the purification protocol is not always critical, but for certain experiments it is crucial that the enzyme not be bound to undesired ligands. Previously described methods for the expression and purification of DHFR from various sources, including *E. coli* DHFR (ecDHFR) and human DHFR (hDHFR) [7,8,9,10], rely on methotrexate (MTX) affinity or ion exchange chromatography to isolate the enzyme in the native state, often bound to folate (FOL) or dihydrofolate (DHF). Bound ligands are then removed by extensive dialysis or isoelectric focusing [7]. For NMR experiments that focus on characterizing differences between intermediates in the DHFR catalytic cycle, it is imperative that the samples contain not even trace amounts of contaminating ligands. After MTX affinity purification, complete removal of ligands is not easily achieved for hDHFR, and isoelectric focusing is not efficient at the scale required for structural studies and is often not easily accessible.

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Our detailed NMR studies of human and *E. coli* DHFR complexes prompted us to evaluate the nature of ligands bound to DHFR produced via different purification methods. Depending on the desired enzyme complex and experimental plan, some purification methods are more reliable than others, and can potentially impact the results. Certain ecDHFR and hDHFR complexes can only be obtained at a high level of purity by refolding apo-enzyme prepared using reversed-phase HPLC purification in the presence of the desired ligand. Interestingly, our experiments also reveal the preferred ligands of both human and *E. coli* DHFR in *E. coli* cells; while human DHFR is tightly bound to NADP⁺ or NADPH in the cell, its *E. coli* counterpart is bound with high affinity to THF. We found this intriguing, given the high structural homology of the two enzymes, and pursued it further. We show that a humanized mutant of ecDHFR, N23PP/S148A ecDHFR, is tightly bound to NADP instead of THF. Since the conformation of ecDHFR is known to be ligand dependent, our data yields useful information on the most prevalent conformational state in a cellular context, which may be of use for drug design.

Materials and Methods

hDHFR and ecDHFR were overexpressed and purified as described in the Supporting Material and as previously reported [8]. Two purification strategies were used for hDHFR and ecDHFR: a native purification strategy using anion exchange followed by size exclusion chromatography, and a novel reversed-phase HPLC strategy followed by refolding the pure protein. Refolding of hDHFR can be optimally achieved by rapid dilution, as the protein does not refold efficiently at high concentrations. Briefly, lyophilized hDHFR and ecDHFR were solubilized in 8M urea, and refolded by diluting dropwise into buffer without urea and with 10-fold excess ligand, with constant gentle stirring. hDHFR can be refolded most efficiently at a final protein concentration of $\leq 10 \mu\text{M}$, while ecDHFR can be refolded at a protein concentration of up to $100 \mu\text{M}$. A detailed description of the refolding method is provided in the Supporting Material. Kinetic measurements were made as described in the Supporting Material and Fig. S1. Standard ¹H-¹⁵N HSQCs were recorded at 750 MHz or 600 MHz, at a temperature of 300 K.

Results and Discussion

Human DHFR is optimally stable when expressed in the presence of folic acid

Our expression protocol for hDHFR differs from previously published methods in one significant aspect: the cells are grown in the presence of 2mM folic acid (folate). Comparison of the ¹⁵N-HSQC spectra of hDHFR in cell lysates derived from cells grown in medium with and without added folate clearly indicates that hDHFR expressed in the presence of folate is well folded and stable, with well dispersed peaks in the proton dimension, whereas hDHFR expressed in the absence of folate is largely aggregated or misfolded (Fig. 1). Subsequent addition of either folic acid or NADP⁺ to hDHFR expressed without folate does not result in any change of the spectrum shown in Fig. 1a, indicating that once the protein is misfolded and/or aggregated, it cannot be recovered. This result shows that under our experimental conditions, hDHFR overexpressed in *E. coli* folds optimally and is stable only when the medium is supplemented with excess folate, yielding higher recovery of hDHFR per liter of labeled medium than previously reported [7].

Natively purified and refolded ecDHFR have identical ¹H-¹⁵N HSQC spectra and activity

Prior to employing the reversed-phase HPLC and refolding method to human DHFR, we tested this method on ecDHFR, as ecDHFR is more robust than hDHFR. Complexes of ecDHFR (purified under native conditions as previously reported [8]) corresponding to all stable intermediates in the catalytic cycle were prepared and ¹H-¹⁵N HSQC spectra were

recorded. For ecDHFR:NADP⁺:FOL, ecDHFR:NADP⁺:THF, ecDHFR:NADPH:THF and ecDHFR:THF, the ¹H-¹⁵N HSQC spectra are identical for samples made using either purification method, showing that the reversed-phase purification method followed by refolding works well (Fig. 2), and also indicating that either method can be used to reliably prepare these complexes. In addition, we measured the activity of ecDHFR prepared under native and refolded conditions, and show that the enzyme activity is unchanged by refolding *in vitro* (Fig. S1).

Purification of human DHFR under native conditions yields protein with endogenously bound NADP

The purity of hDHFR resulting from our native purification strategy involving anion exchange and gel filtration chromatography is suitable for NMR spectroscopy, and the sample produces excellent ¹H-¹⁵N HSQC spectra (Fig. 3a). However, this protocol yields protein that is bound to NADP, despite the fact that exogenous NADP was not added at any step during expression or purification. hDHFR binds a mixture of endogenous NADP⁺ and NADPH in the cell. Bound NADPH will be converted to NADP⁺ in the presence of folic acid; therefore our samples are contaminated with NADP⁺. However, due to the ambiguity of the endogenously bound ratio of NADP⁺/NADPH, we refer to the bound cofactor simply as NADP. Spectra of natively purified hDHFR show that addition of excess NADP⁺ to the “hDHFR:FOL” complex does not result in the large chemical shift changes expected upon formation of the ternary hDHFR:NADP⁺:FOL complex. Amide chemical shifts are highly sensitive to chemical environment; therefore, addition of a ligand should result in large chemical shift changes, especially in the ligand-binding site.

Given this perplexing result, we hypothesized that hDHFR prepared by our native purification strategy contains endogenously bound NADP, and thus further addition of NADP⁺ does not result in any chemical shift changes in the ¹H-¹⁵N HSQC spectrum. This was confirmed by isolation of apo-hDHFR using our novel reversed-phase HPLC purification strategy. HPLC purified apo-hDHFR refolded in the presence of NADP⁺ and folic acid has an identical ¹H-¹⁵N HSQC spectrum to that of natively purified hDHFR bound to endogenous NADP and excess folate (Fig. 3b).

Under our natively purified conditions, extensive dialysis is not sufficient to remove the ligands from the ternary complex (data not shown). This purification method is only suitable for obtaining the hDHFR:NADP⁺:FOL complex. Using samples prepared with apo-hDHFR purified by reversed-phase HPLC, it is possible to compare the spectra of the binary hDHFR:FOL and ternary hDHFR:NADP⁺:FOL complexes (Fig 3c). As for ecDHFR, the spectra of the natively purified and refolded ternary complexes are identical (data not shown) and the activity of the enzyme is maintained (Fig. S1). The spectrum of the binary hDHFR:FOL complex is well dispersed and of uniform intensity. Several crosspeaks exhibit large chemical shift changes between the true hDHFR:FOL complex and the hDHFR:NADP⁺:FOL ternary complex. As expected, these resonances correspond to residues in the NADP binding site.

Purification of *E. coli* DHFR under certain native conditions shows the presence of endogenously bound THF

Analysis of several spectra revealed that ecDHFR samples prepared from protein purified using the ion exchange-size exclusion native purification strategy were contaminated with THF (Fig. 4). As no THF is added at any step of the procedure, we conclude that THF endogenous to the *E. coli* cells binds the enzyme during overexpression, and remains bound throughout the purification. Based on the intensity of minor peaks in the natively purified ecDHFR:NADPH spectra, which correspond directly to the ecDHFR:NADPH:THF

resonances, we conclude that ~5-7% of purified ecDHFR contains bound THF. The percentage of bound THF is dependent on, amongst other things, the protein yield and total induction time. The residual THF cannot be removed easily, even by extensive dialysis. Purification of WT ecDHFR using MTX affinity chromatography will not result in this contamination since ecDHFR bound to THF cannot bind the MTX affinity column. We therefore conclude that for studies on *E. coli* DHFR complexes without THF, purification by reversed-phase HPLC or traditional MTX affinity purification [9] is preferred.

Designed ecDHFR mutant switches binding specificity in the cell

Natively purified hDHFR contains endogenously bound NADP, while natively purified ecDHFR (by the ion exchange-size exclusion method) contains endogenously bound THF. We evaluated the endogenous ligand binding propensities of an ecDHFR mutant (N23PP/S148A ecDHFR), in which the active site loops of the *E. coli* enzyme were “humanized” by substitution of residues from the human sequence to lock the enzyme in a closed conformation [11]. The ^1H - ^{15}N HSQC of natively purified N23PP/S148A ecDHFR expressed without exogenous ligands added to the medium (Fig. 5a), shows the presence of multiple conformations and/or ligand bound species that likely correspond to the apo enzyme and E:NADP. Addition of excess NADPH to this sample resolves the heterogeneity and yields a single set of peaks corresponding to the E:NADPH complex (Fig. 5b). Addition of folate to the sample shown in Fig. 5a results in a ^1H - ^{15}N HSQC with two sets of peaks, likely corresponding to E:FOL and E:NADP⁺:FOL complexes (Fig. 5c,d). Unlike wild type ecDHFR, the ^1H - ^{15}N HSQC of N23PP/S148A with added NADPH shows only a single set of resonances with uniform intensity, confirming that endogenous THF is not present and that the “humanized” *E. coli* mutant shows a similar specificity for endogenous ligands as hDHFR.

Conclusions

Our work addresses two issues that are relevant to further studies on DHFR. First, our data reveal the presence and identity of endogenously bound ligands to hDHFR and ecDHFR expressed in *E. coli*. Second, since the presence of these endogenously bound ligands might not be desirable for certain experiments, we present suitable purification strategies for obtaining pure samples of human and *E. coli* DHFR.

For the purpose of detailed NMR and other structural studies, it is important to generate samples that contain only the desired ligands. Our data show that for wild type hDHFR, any native purification strategy will likely result in contamination with NADP. When hDHFR is expressed in the presence of folic acid, which boosts the yield of properly folded protein, a ternary complex (hDHFR:NADP⁺:FOL) is formed. ecDHFR, on the other hand, is contaminated with THF after anion exchange and size exclusion chromatography. Neither contaminant can be removed by extensive dialysis following native purification.

While, in general, native purification strategies are preferred in order to maintain native structure and enzyme activity, we have shown that in the case of human and *E. coli* DHFR, highly pure, homogeneous and active enzyme complexes are optimally prepared by refolding reversed-phase HPLC purified apo-enzyme in the presence of the desired ligands. A partial refolding strategy has been described [12] for preparation of folate-free ecDHFR, but this method has yet to be applied to enzymes with less robust refolding properties, such as hDHFR for which refolding under less than optimal conditions results in considerably reduced yields [13,14]. For both DHFRs, purification by reversed-phase HPLC yields protein that is ligand-free, and that can efficiently be refolded into the desired complex at the scales required for structural studies and drug development. hDHFR NMR samples have previously been prepared by expression of hDHFR in the presence of trimethoprim,

followed by methotrexate affinity purification, and without isoelectric focusing, which is usually carried out to remove bound ligands [15]. Binary hDHFR :MTX complexes prepared in this manner did not contain observable bound NADP. However, this work (15) used a different cell line, with likely different endogenous ligand concentrations, for protein expression. Additionally, the protein was stabilized using trimethoprim (TMP) in the medium instead of folate. The affinity of NADP is higher for hE:FOL than for apo hE [5], and the affinity of FOL for hE is higher than that of TMP for hE [16]. It follows that since the affinity of NADP for hE:FOL is higher than for hE:TMP, NADP likely binds with higher affinity when the enzyme is expressed in the presence of FOL than in the presence of TMP, and thus copurifies when the enzyme is expressed in the presence of FOL. It is therefore important to note that depending on the affinity and cooperativity of binding, and the ligand used for stabilization during expression, different ligands might copurify with the enzyme.

The presence of endogenously bound ligands after native DHFR purification has been considered in the past during preparation of DHFR. However, the identity of the bound ligands remained unknown. Our results show that while ecDHFR is preferentially bound to THF, and does not co-purify with endogenous NADP, hDHFR co-purifies with endogenous NADP. Moreover, a mutant of ecDHFR designed based on the human sequence apparently switches binding affinities to resemble the human enzyme, and correspondingly, is purified bound to endogenous NADP and not THF. The differential ligand-binding properties of ecDHFR, hDHFR and mutant ecDHFR are consistent with reported dissociation constants; hDHFR binds NADP with higher affinity than ecDHFR does (for hDHFR, $K_d = 2.3 \mu\text{M}$ for NADP^+ , $K_d = 0.05 \mu\text{M}$ for NADPH, while for ecDHFR $K_d = 24 \mu\text{M}$ for NADP^+ , $K_d = 0.33 \mu\text{M}$ for NADPH) [3,5,17,18]. The dissociation constants for these ligands are in the high nanomolar-low micromolar range, and are for the apo enzymes. As has been shown before, there is some cooperativity in ligand binding [5]; therefore, it is likely that the affinities of these ligands are somewhat higher in the cell lysate. It is interesting to note that a large number of structures deposited in the PDB contain unexpected copurifying ligands, including 65% of the structures solved by the Protein Structure Initiative (PSI) [19]. These ligands can be acquired at the expression stage (as in the case of DHFR), or at any of the purification or crystallization stages, as buffer components can often mimic ligands. As seen in the case of DHFR, the co-purifying ligands need not bind with particularly high affinity, and the dissociation constants can be in the low micromolar range. Since several proteins, including ecDHFR, exhibit ligand-dependent conformational changes, an understanding of endogenously bound ligands may yield insights into protein conformations in the cellular environment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Endogenous ligands bound to the enzyme dihydrofolate reductase (DHFR) were identified by 2D NMR for human DHFR (hDHFR) and *E. coli* DHFR (ecDHFR) expressed in bacterial cells.
- ¹⁵N HSQC analysis shows that hDHFR is preferentially bound to NADP, while ecDHFR is preferentially bound to THF.
- A designed “humanized” mutant of ecDHFR switches binding specificities to resemble the human enzyme.
- Novel purification schemes are introduced for both DHFRs to obtain pure samples without traces of endogenously bound ligands.

Figure 1. hDHFR is optimally folded and stable when expressed in the presence of folic acid. (A) ^1H - ^{15}N HSQC of hDHFR lysate, expressed without folic acid. (B) ^1H - ^{15}N HSQC of hDHFR lysate, expressed with 2 mM folic acid.

Figure 2.

^1H - ^{15}N HSQC spectra for ternary complexes of *E. coli* DHFR show that ecDHFR can be refolded successfully. Spectra of natively purified protein (black) and refolded HPLC purified protein (red) are shown for: ecDHFR:NADP⁺:FOL (A) and ecDHFR:NADP⁺:THF (B).

Figure 3.

NADP co-purifies with hDHFR under native purification conditions. (A) ^1H - ^{15}N HSQCs of hDHFR purified using anion exchange followed by gel filtration, with addition of excess folic acid (black) and with addition of excess NADP^+ and folic acid (red). (B) ^1H - ^{15}N HSQC spectra of hDHFR purified under native conditions with addition of excess folic acid (black) and hDHFR purified by reversed-phase HPLC and refolded with addition of excess NADP^+ and folic acid (magenta) are almost identical, showing that hDHFR can be successfully refolded. (C) ^1H - ^{15}N HSQC spectra of hDHFR: NADP^+ :FOL (red) and hDHFR:FOL (blue) prepared by refolding HPLC purified apo-hDHFR with the respective ligands.

Figure 4. ecDHFR:NADPH binary complex is contaminated with bound THF. (A) ^1H - ^{15}N HSQC of ecDHFR purified using anion exchange and gel filtration, with addition of excess NADPH. Several minor peaks are visible, indicating the presence of another species. (B) Overlay of A (black) with ecDHFR:NADPH prepared by HPLC purification (red) and ecDHFR:NADP⁺:THF (cyan). The sample prepared using HPLC purification (red) does not contain the minor peaks observed in A. The minor peaks can be accounted for by the ecDHFR:NADP⁺:THF (cyan) spectrum. The minor peaks arise from contamination with endogenous THF, which remains bound to ecDHFR throughout the anion exchange and gel filtration purification steps.

Figure 5.

N23PP/S148A ecDHFR is bound to endogenous NADP after native purification by ion exchange and size exclusion chromatography. (A) ^1H - ^{15}N HSQC of natively purified N23PP/S148A ecDHFR without addition of any ligand (black), showing several more resonances than residues in the protein. Upon addition of NADPH to this sample, the E:NADPH complex is formed (red), in which a clean spectrum of uniform intensity is obtained, with one resonance per residue. The E:NADPH spectrum overlays almost perfectly with a subset of the peaks in the black spectrum, showing that NADP is present in ~50% of the natively purified protein sample without any exogenous ligand added. (B) ^1H - ^{15}N HSQC of natively purified mutant protein with added folate (black) exhibits more resonances than expected for a uniform E:FOL complex. Overlay of this spectrum with E:NADP $^+$:FOL (red) shows that endogenously bound NADP is present in the natively purified sample.