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Letter to the Editor: Backbone H^N, N, C^α, C' and C^β assignments of the 19 kDa DHFR/NADPH complex at 9 °C and pH 7.6

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Biological context

Dihydrofolate reductase (DHFR) is an enzyme that uses NADPH to reduce folic acid (folate) to 7,8-dihydrofolate (H_2F) and H_2F to 5,6,7,8-tetrahydrofolate (H_4F). Tetrahydrofolate is a versatile enzyme cofactor that functions to transfer one-carbon units in several oxidation states: formate, formaldehyde and methanol. The inhibition of DHFR blocks several H_4F dependent biological reactions such as the synthesis of thymidalate, purines, histidine and methionine. DHFR is therefore a very attractive drug target. The kinetics of wild type *E. coli* DHFR has been extensively investigated, resulting in a complete description of the overall kinetic pathway (Fierke et al., 1987). The goal of our project is to use NMR relaxation data to obtain novel experimental information on the intrinsic molecular dynamics of this important enzyme and how they are modulated by the interactions with substrate, cofactor and product at the various stages in the catalytic cycle.

We focused on the binary complex between DHFR and NADPH, which is the precursor of the Michaelis complex. NADPH is not stable at acidic conditions: a pH lower than 5 leads to a rapid loss of the characteristic UV absorption of the nicotinamide ring at 340 nm (Oppenheimer, 1982). Because of greater NADPH stability and better water solubility, most spectroscopic studies have been performed at pH higher than 8. NADPH reacts with oxygen to form $NADP^+$, and it is known to be involved mainly in metabolite reduction. It was recently reported that DHFR can catalyze the oxidation of NADPH to $NADP^+$ even in the absense of a reducible substrate such as folate or H_2F (Hsu et al., 1998). In our case, the instability of the complex was noted during the preliminary NMR experiments as a severe decrease in the observed signal of the ^{15}N-HSQC spectrum on the time-scale of hours.

We adopted several approaches to slow down NADPH oxidation so that the sample would not undergo any detectable change or degradation during the time needed for NMR relaxation measurements. These approaches are reported here, together with the backbone resonance assignments of the DHFR/NADPH complex.

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Methods and results

E. coli BL21(DE3) competent cells were transformed with a pET-22b plasmid containing the *E. coli* K-12 strain wild type DHFR gene. Cells were grown at 37 °C in minimal medium containing (^{15}N) ammonium sulfate and (^{13}C) glucose as the only source of nitrogen and carbon, and overexpression was induced by IPTG when cells reached $OD_{600} = 1$. The enzyme was purified from the soluble fraction using a variation of the protocol of Falzone et al. (1994).

A 2D ^{15}N-HSQC experiment was used to monitor sample stability and to optimize solution conditions for NMR experiments. Probe head temperature was calibrated using a neat methanol sample and controlled to within ±0.1 °C. We used enzymatically reduced NADPH (Sigma), following reports (Rafter and Colowick, 1957) that it is less acid labile than the chemically reduced material. The highest pH at which there was no major intensity loss of the NMR signal (due to amide-proton/solvent exchange) was 7.6. At this pH, and with a phosphate buffer concentration of

70 mM, the maximum cofactor concentration that can be buffered is 40 mM NADPH. Based on a 40× excess of cofactor relatively to the protein, the DHFR concentration was then limited to 1 mM. This NMR buffer (70 mM KPi, 25 mM KCl, 7% D_2O, 0.02% NaN_3, 1 mM DTT, 20 μM DSS) was deoxygenated by several freeze-pump-thaw cycles in a vacuum manifold line and transferred to a glove box flushed with argon. In the glove box, DHFR in 50 mM NaPi buffer was added to a desalting column pre-equilibrated with the deoxygenated NMR buffer and then eluted in the new buffer. This solution was used to dissolve the solid NADPH. After preparation of the complex sample in the deoxygenated NMR buffer, the pH was adjusted to 7.6 at 20 °C and the sample was filtered through a 0.2 μm filter. Samples were immediately transferred to custom-made amberized tubes (NADPH is very light sensitive) with teflon vacuum valves (Wilmad Glass). After being checked in the NMR spectrometer, the NMR tubes were connected to the vacuum manifold. The sample was once more frozen, pumped and thawed under argon pressure of approximately 0.25 atm. The tubes were then flame-sealed and stored at 4 °C to assure maximum sample stability.

NMR spectra were acquired at 500 and 600 MHz proton frequencies, on Bruker AMX, AMX-II or DRX spectrometers. Data were processed using NMRPipe and analyzed with NMRView using additional in-house scripts. Chemical shifts were referenced directly/indirectly from the proton frequency of the DSS resonance at 0.00 ppm. At a probe temperature of 9 °C, a ^{15}N labeled DHFR sample was stable for more than 3 weeks.

Backbone assignments were made using HNCA, HNCACB, CBCA(CO)NH, HNCO, HN(CA)CO and ^{15}N HSQC-NOESY-HSQC 3D datasets acquired on a $^{13}C/^{15}N$ labeled sample. The processed 3D spectra were used to establish sequence connectivity between the (H^N, ^{15}N) pairs with the help of a dual strip tool in NMRView. Stretches of connected pairs were sequence specifically assigned using a modified version of the seq_prob program (Grseziek and Bax, 1993). Due to the relatively high molecular weight of the complex (>20 kDa for the double labeled DHFR) and to the experimental conditions needed for sample stability (9 °C and pH 7.6), we observed severe signal broadening (attenuation) due to rapid transverse relaxation during the long carbon evolution times.

In order to overcome this problem, we expressed and purified triple-labeled ($^2H/^{13}C/^{15}N$) DHFR by growing the BL21(DE3) cells in 100% D_2O using the same protocol as described above. We acquired deuterium decoupled versions of HNCA, HN(CO)CA, HN(CA)CB and HN(COCA)CB 3D experiments following the protocol given by Yamazaki et al. (1994). Each pair enabled us to uniquely assign the inter- and intra-residue resonances.

Extent of assignments and data deposition

Since the resonance frequencies for the amide (H^N,^{15}N) pairs are not changed by deuteration, we were able to transfer the established connectivities to the protonated protein complex. H^N and ^{15}N assignments of the 2D ^{15}N-HSQC backbone peaks were obtained for all but one (Ile^{14}) of the expected 148 residues (all but the 10 prolines and Met^1). Protonated C^α and C^β assignments were obtained for 158 residues (all but Pro^{55}). Carbonyl C′ chemical shifts were assigned for 139 residues based on the HNCO spectrum. Besides the 10 residues preceding the prolines and the C-terminal Arg^{159}, carbonyl shifts could not be assigned for five other residues: Met^1, Val^{13} (which is followed by Ile^{14}), Met^{16}, Asn^{34} and Ala^{107}. The assignments have been submitted to the BMRB databank (BMRB-4554).

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