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Effects of Co-operative Ligand Binding on Protein Amide NH Hydrogen Exchange

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Amide protection factors have been determined from NMR measurements of hydrogen/deuterium amide NH exchange rates measured on assigned signals from *Lactobacillus casei* apo-DHFR and its binary and ternary complexes with trimethoprim (TMP), folinic acid and coenzymes (NADPH/$NADP^+$). The substantial sizes of the residue-specific ΔH and $T\Delta S$ values for the opening/closing events in NH exchange for most of the measurable residues in apo-DHFR indicate that sub-global or global rather than local exchange mechanisms are usually involved. The amide groups of residues in helices and sheets are those most protected in apo-DHFR and its complexes, and the protection factors are generally related to the tightness of ligand binding. The effects of ligand binding that lead to changes in amide protection are not localised to specific binding sites but are spread throughout the structure *via* a network of intramolecular interactions. Although the increase in protein stability in the DHFR.TMP.NADPH complex involves increased ordering in the protein structure (requiring $T\Delta S$ energy) this is recovered, to a large extent, by the stronger binding (enthalpic ΔH) interactions made possible by the reduced motion in the protein. The ligand-induced protection effects in the ternary complexes DHFR.TMP.NADPH (large positive binding co-operativity) and DHFR.folinic acid.NADPH (large negative binding co-operativity) mirror the co-operative effects seen in the ligand binding. For the DHFR.TMP.NADPH complex, the ligand-induced protection factors result in $\Delta\Delta G_o$ values for many residues being larger than the $\Delta\Delta G_o$ values in the corresponding binary complexes. In contrast, for DHFR.folinic acid.-NADPH, the $\Delta\Delta G_o$ values are generally smaller than many of those in the corresponding binary complexes. The results indicate that changes in protein conformational flexibility on formation of the ligand complex play an important role in determining the co-operativity in the ligand binding.

Keywords: hydrogen exchange; dihydrofolate reductase; protein–ligand interactions; co-operative binding; NMR

Introduction

Detailed information about fluctuations in protein structures can be obtained from studies of amide NH protection factors detected *via* NMR-determined measurements of NH hydrogen/deuterium exchange (H/D) in proteins. Such fluctuations expose some of the NH protons to the aqueous solvent, thus facilitating the NH exchange process. This approach has been shown to be useful for assessing the stability of specific hydrogen bonds within a protein, and for monitoring the effects of ligand binding.[1] For several proteins, it has been proposed that the most highly protected NH groups exchange by means of a global unfolding process.[1–7] In these cases the change in free energy ΔG_o for the opening/closing equilibrium associated with the protected amide NH has a value approaching that of the ΔG for global unfolding. However,

Abbreviations used: H/D, hydrogen/deuterium exchange; DHFR, dihydrofolate reductase; folinic acid 5-formyl-5,6,7,8-tetrahydrofolic acid; HSQC, hetero-nuclear single quantum coherence spectroscopy; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; S.E.M., standard error of the mean; TMP, trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine).

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observations of different ΔG_o values (related directly to protection factors) for many NHs in a protein indicate that local and sub-global unfolding mechanisms are responsible for the exchange in most cases. The emerging picture is one where the NH exchange events take place within a complex statistical ensemble of different conformational states.[8–17] Studies of the dependence of NH exchange rates on temperature and chemical denaturant concentration,[18,19] and on hydrostatic pressure,[20] have been used to identify residues for which the exchange is controlled by local rather than global or sub-global mechanisms. Such information has been used to define regions of co-operative stability. However, Clarke and Fersht have cautioned against using this approach in isolation for determining folding pathways.[21] Several workers have discussed the implications of NH exchange studies in relation to co-operative interactions within protein structures.[14,22,23] Calculated ligand-induced NH protection factors have been used to attempt to indicate the protein structural pathway followed by the stabilising interactions accompanying ligand binding.[24] Other workers have used NH/ND exchange data to explore the origins of co-operative binding,[25–27] and to reveal details of the order of events involved in protein–DNA recognition.[28,29]

Here, we report on the NH protection factors for apo-dihydrofolate reductase (DHFR) and a series of its binary and ternary ligand complexes that are known to show binding co-operativity. DHFR is a pharmacologically important target enzyme for several antibacterial, anticancer and antimalarial drugs. These antifolate drugs often bind to the protein with large positive co-operativity in the presence of the coenzyme NADPH.[30] In the case of the antibacterial drug trimethoprim (TMP; trimethoprim(2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine)), the co-operative binding has been implicated directly in the specificity of drug binding to bacterial DHFRs when compared to mammalian DHFRs.[31] Another ligand, the tetrahydrofolate analogue 5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid), binds to DHFR with strong negative co-operativity in the presence of NADPH:[32] similar negative co-operativity binding effects have important implications in the control of product release in the enzyme reaction mechanism.[33] The detailed origins of positive and negative co-operative ligand binding are still poorly understood. One possible explanation calls for the first ligand to induce conformational changes in the protein that produce an altered binding environment for the second ligand. In the present study, measurements of NH exchange protection factors for *Lactobacillus casei* DHFR, and its binary and ternary complexes with trimethoprim, folinic acid and coenzyme are examined to explore the thermodynamics of the ligand-induced conformational changes.

Results

NH protection factors in DHFR and its complexes

The ^{15}N–^{1}H heteronuclear single quantum coherence spectroscopy (HSQC) spectra at 15 °C and pH 6.5 of apo-DHFR dissolved in H_2O and after 20 h of H/D in $^{2}H_2O$ are shown in Figure 1(a) and (b), respectively. Even at 15 °C, relatively few protein residues remain unexchanged in the $^{2}H_2O$ spectra. Most of the unchanged residues belong to amide NHs in the well-protected β-strand protein core of DHFR (the structure has an eight-stranded twisted β-sheet and four α-helices as shown in Figure 2).[34] Upon ligand binding, the amide NH groups become more protected, as seen by comparing the spectra of apo-DHFR (Figure 1(b)) with those of the DHFR.TMP and DHFR.TMP.NADPH complexes at pH 6.5 (Figure 1(c) and (d)). For apo-DHFR it was possible to measure only 50 NH resonances, whereas for the DHFR.TMP and DHFR.TMP.NADPH complexes there were 83 and 91 measured NH resonances, respectively (including some from residues in non-regular secondary structure elements).

The measurements of amide H/D rates in ^{15}N-labelled apo-DHFR and its complexes were carried out using heteronuclear ^{15}N–^{1}H NMR spectroscopy (at 15 °C and pH 6.5) and the protection factors were determined as ΔG_o values, the Gibbs energy differences between the open and closed states (see Tables 1 and 2). The exchange rates for apo-DHFR were measured over the range 5–20 °C and the ΔG_o, ΔH and $T\Delta S$ values together with the specific heat capacity difference (ΔC_p) values for the opening/closing events for the individual residues were extracted from the data as described in Materials and Methods (see Table 3). The ΔG_o values measured at 15 °C and pH 6.5 for apo-DHFR and its binary and ternary complexes with TMP, folinic acid and NADPH are given in Table 1: most of the measurable protection factors (ΔG_o values) are for amide NH protons located either in or close to the well-defined secondary structure elements shown in Figure 2. Table 1 contains the ligand-induced protection factors expressed as $\Delta\Delta G_o$ values for all residues for which data for apo-DHFR and the relevant complexes are available. Similar data for the binary and ternary complexes with TMP and $NADP^+$ are given in Table 2. Bar charts indicating the ligand-induced protection factors ($\Delta\Delta G_o$ values) for all the complexes are given in Figure 3.

DHFR complexes have larger overall NH protection factors (higher ΔG_o values) than apo-DHFR, where the protected residues are mainly in the eight β-strands (strands A–H) and α-helices (C and E) (see Table 1). For the binary complexes with TMP, folinic acid, NADPH and $NADP^+$ there are substantial increases in the protein protection factors for many of the residues. The NH reso-