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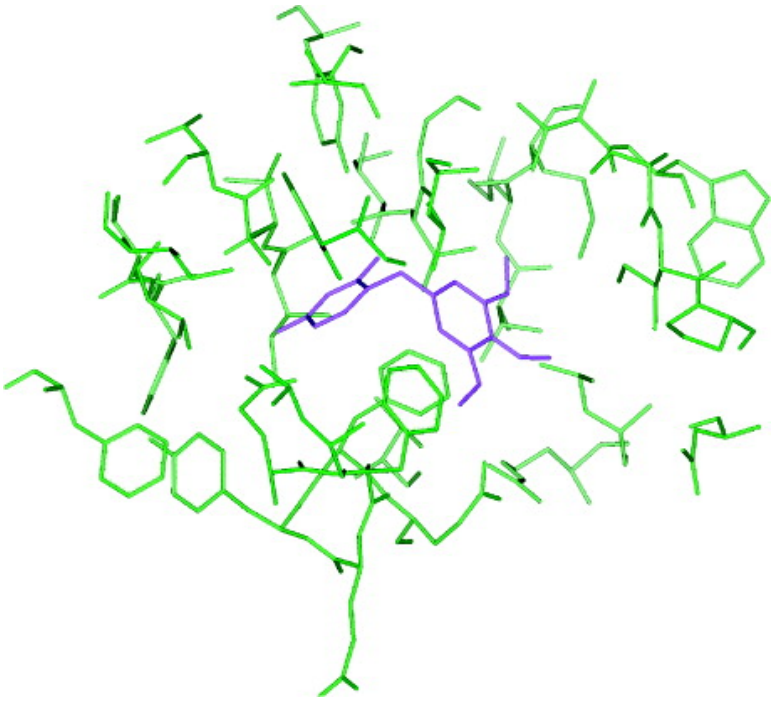

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Determination of the Conformation of Trimethoprim in the Binding Pocket of Bovine Dihydrofolate Reductase from a STD-NMR Intensity-Restrained CORCEMA-ST Optimization

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Abstract: Dihydrofolate reductase (DHFR) is a pharmacologically important intracellular target enzyme for folate antagonists, including the antibacterial agent trimethoprim (TMP). The structures of DHFR from various sources with and without the bound ligands have been determined by X-ray crystallography and solution NMR spectroscopy. However, there is no crystal or solution NMR structure for the bovine DHFR/TMP complex. Here we report the solution structure of TMP within the binding pocket of bovine DHFR using a novel method developed in our laboratory, viz., STD-NMR intensity-restrained CORCEMA-ST optimization (SICO) utilizing experimental STD data on this complex, and demonstrate that its solution structure is essentially identical to the one in the crystal structure of the homologous chicken liver DHFR/TMP complex. The excellent agreement we obtain between the experimental and predicted STDs also serves as a validation of the CORCEMA-ST methodology.

1. Introduction

Dihydrofolate reductase (DHFR) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate. Tetrahydrofolate or its derivatives are essential cofactors in the biosynthesis of purine nucleotides, thymidylate, and several amino acids.[1] Failure to maintain adequate levels of tetrahydrofolate results in increased nucleic acid synthesis and cessation of cell growth. On the basis of metabolic consequences, DHFR is a pharmacologically important intracellular target enzyme for a number of folate antagonists, including the antibacterial agent trimethoprim (TMP).[2,3] The structure of DHFR from various sources with and without the bound ligand has been determined by X-ray crystallography[4−6] and solution NMR spectroscopy.[7−9] However, there is no solution or crystal structure for the TMP complex of bovine DHFR. In this work, we report the bound conformation of TMP within the binding pocket of bovine DHFR using a novel method developed in our laboratory, viz., STD-NMR intensity-restrained CORCEMA-ST optimization (SICO)[10,11] utilizing experimental STD data on this complex.

STD-NMR spectroscopy represents one of the most sensitive and popular NMR methods for screening compound libraries.[12,13] This technique is frequently used to analyze ligand−receptor binding and thus provide a means of assessing the molecular interactions in biomolecular complexes. Recently, our laboratory has developed CORCEMA-ST and SICO procedures[10,11] for the quantitative determination of bound ligand conformation within the binding pocket of the target protein using STD-NMR data and extended the scope of this STD method beyond its current application in compound library screening[12,14] and in the qualitative epitope mapping[15−17] of ligands bound to protein targets. We have also illustrated the application of the method on complexes with known crystal structures where the STD data have been utilized to refine the solution conformation of the bound ligand within the protein binding pocket.[18,19] In this contribution we report the bound conformation of the antibacterial agent TMP within the binding pocket of bovine DHFR for which there is no crystal structure

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yet, using the SICO procedure[10,11] and experimental STD-NMR data.[20] By combining the crystallographic data on chicken liver DHFR/TMP complex, NMR assignments on human DHFR/methotrexate complex, and the experimental STD NMR data on bovine DHFR/TMP complex, we were able to determine the structure of the bound TMP with in the bovine DHFR binding pocket. Our work also underscores the importance of a quantitative interpretation of the STD intensities by CORCEMA analysis on available initial models (either from X-ray crystallography or any model generated by computational docking program) for rigorous structural calculations.

2.Methods

CORCEMA-ST Procedure. The CORCEMA-ST program was written in Matlab. The underlying theory of CORCEMA-ST has been described.[10,11] This program calculates the expected STD-NMR intensities for any proposed molecular model of a ligand−receptor complex using parameters such as the correlation times, knowledge of saturated protein protons, exchange rates, and spectrometer frequency. The expression for observable magnetization in a STD experiment by assuming infinite delay between each scan is given by[10]

$$\mathbf{I}(t) = \mathbf{I}_0 + [\mathbf{1} - \exp\{-\mathbf{D}t\}]\mathbf{D}^{-1}\mathbf{Q} \qquad (1)$$

where $\mathbf{I}(t)$ is a column matrix containing the magnetizations for the ligand and for those protein protons that do not experience a direct rf saturation in their free and bound states. $\mathbf{I}_0$ is the corresponding thermal equilibrium magnetization matrix. **Q** is a column matrix containing cross-relaxation terms between the protein protons that experience a direct rf saturation and the rest of the protein protons and the bound ligand protons. The dynamic matrix **D** is a square matrix and is the sum of the relaxation rate matrix **R** and a kinetic matrix **K**, both of reduced dimensions. These matrixes have been defined earlier.[10] t is the time period for which protein protons experience rf irradiation. The CORCEMA-ST program also has a provision for taking into account the effect of finite delays (t_d) between scans in calculating STD effects, and this finite delay was taken into account in the current analysis. To speed up the computation of **R** matrix, spectral densities are usually calculated for only those proton pairs having a distance of 10Å or less. To reduce the dimensions of the matrixes to a manageable size, only those protein residues within a specified distance (usually 7Å to 10Å) from the ligand are included in the calculations.

The starting model for the CORCEMA calculations can be either an available crystallographic structure for a homologous complex or a model generated by a computer docking program such as AutoDock. The bovine DHFR/TMP complex was modeled from the crystal structure[4] of chicken liver DHFR/TMP−NADPH ternary complex (coordinates received from Dr. D. A. Matthews). The Biopolymer module in Insight-II (Accelrys, Inc) was used to make the necessary amino acid changes in the chicken liver DHFR (Y31F and K32Q) to generate the bovine DHFR complex. We performed an energy minimization of the two substituted residues in the bovine DHFR complex by using the Discover module in Insight-II using steepest descent algorithm and the force field CVFF. This energy-minimized bovine DHFR/TMP complex was used for STD predictions. In the current calculations 35 amino acid residues within a 7Å distance from the ligand were included (I7, V8, A9, V10, I16, L22, W24, L27, E30, F31, Q32, Y33, F34, Q35, T38, V50, I51, M52, T56, W57, S59, I60, P61, N64, L67, R70, I114, V115, G116, Y121, F134, V135, T136, I138, F179). Since STD-NMR measurements were performed in D_2O, we have excluded all exchangeable hydrogens (OH and NH) in our calculations. The STD intensities ($S_{exp,k}$) were calculated as percentage

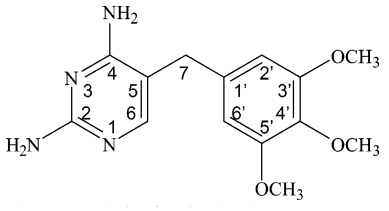

Figure 1. Structure and atom numbering for trimethoprim.

fractional intensity changes ($[(\mathbf{I}_{0k} - \mathbf{I}(t)_k)*100]/\mathbf{I}_{0k}$, where k is a particular proton in the complex, and $\mathbf{I}_{0k}$ its thermal equilibrium value) from the intensity matrix $\mathbf{I}(t)$ and compared to the experimental STD values using an NOE R-factor defined as,[21,22]

$$\text{NOE } R\text{-Factor} = \sqrt{\frac{\Sigma W_k (S_{\text{exp},k} - S_{\text{cal},k})^2}{\Sigma W_k (S_{\text{exp},k})^2}} \qquad (2)$$

In these equations $S_{exp,k}$ and $S_{cal,k}$ refer to experimental and calculated STD values for proton k. The use of a weighting (W_k) proportional to $1/S_{exp,k}$ for each individual STD intensity has the effect of making the R-factor sensitive to significant deviations in small STD values as well as to deviations in the large STD values. In parameter optimizations, the NOE R-factor was used as the target function. In torsion angle refinements, the target function that was optimized was a sum of the NOE R-factor and a van der Waals repulsion term[11,19] which avoids the conflicts between atoms during the rotation across bonds. This repulsion term is defined as[11,23]

$$E_{\text{repel}} = \begin{cases} 0 & \text{if } r \geq s r_{\min} \\ k_{\text{vdw}} (s^2 r^2_{\min} - r^2)^2 & \text{if } r < s r_{\min} \end{cases} \qquad (3)$$

The simulated annealing (SA) refinement, based on the version by Alotto et al.[24] was used for optimizing the parameters to get best fit between the experimental and predicted intensities. It utilizes the standard Metropolis criterion for accepting or rejecting incremental random changes in parameters, one at a time. This method identifies the global minimum within the parameter ranges used in the optimization. In our calculations, the parameters needed for STD predictions are the dissociation constant (K_d), the correlation times for the free ligand (τ_L) and the protein (τ_P), the methyl group internal correlation time (τ_m), the leakage relaxation for all the protons in their free and bound states to mimic nonspecific leakage relaxation with paramagnetic oxygen in the solution, and the order parameter S^2 for methyl group-external proton interactions.[25,26] The association constant value of TMP in complex with DHFR is set at 2×10^7 as determined previously.[27] We have assumed the diffusion-limited on rate as 10^8 s^{-1} M^{-1}. A reasonable value of 0.85 was assumed for S^2. Thus, the remaining parameters needed are τ_P, τ_L, τ_m, and the leakage relaxation. These parameters were determined by SA optimization by using the experimental STD NMR intensities as the constraints. TMP molecule (Figure 1) has three methoxy groups, in which two of them (3′ and 5′ methoxy groups) are symmetrically located; hence, we assumed two different internal correlation times for the methyl groups in the free ligand (τ_{m1} for 4′OMe and τ_{m2} for 3′/5′OMe). An independent calculation showed that variations in the bound methyl internal correlation times do not significantly affect the STD values. Thus, for simplicity we assumed a

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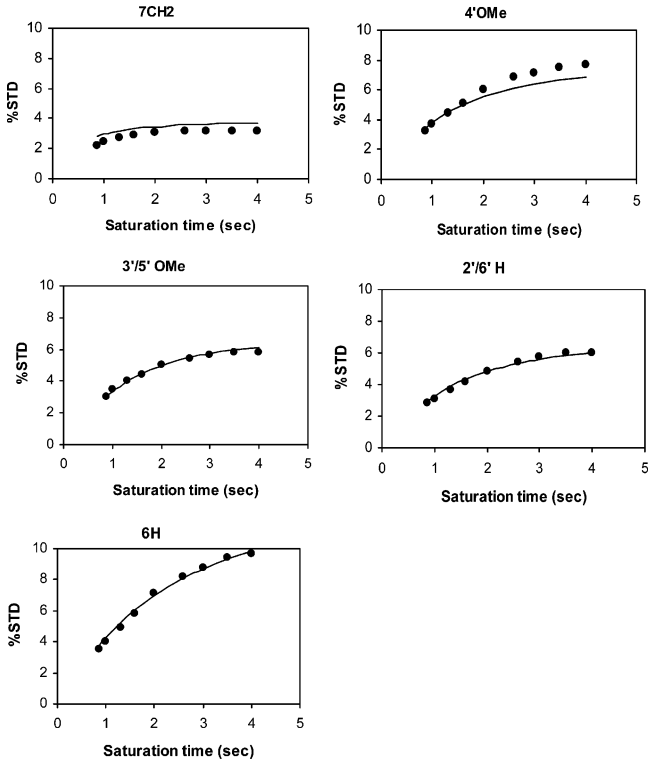

Figure 2. Comparison of experimental STDs (●) and predicted STD values (—) from CORCEMA-ST method for the initial model of the bovine DHFR/TMP complex. The optimized parameters are τ_L: 0.101 ns, τ_P: 20.43 ns; τ_{m1}: 0.81 ps; τ_{m2}: 3.04 ps; τ_{m3}: 3.26 ps; the leakage factor: 0.065 s^{-1}; and the NOE *R*-factor = 0.076.

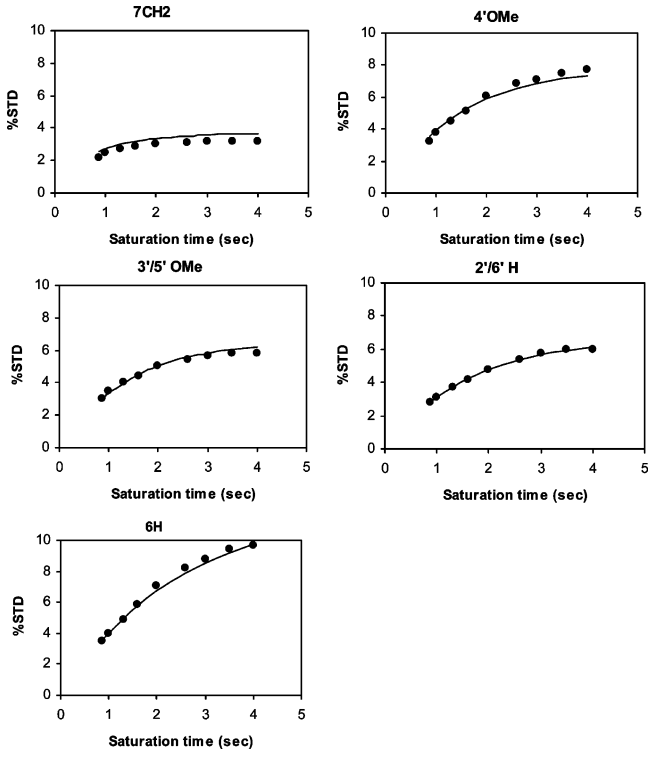

Figure 3. Comparison of experimental STDs (●) and predicted STD values (—) from CORCEMA-ST method for the refined structure ($\theta 1 = -80.46°$ and $\theta 2 = 99.99°$) of bovine DHFR/TMP complex. The optimized parameters are; τ_L: 0.082 ns; τ_P: 20.6 ns; τ_{m1}:0.5 ps; τ_{m2}: 3.96 ps; τ_{m3}: 4.04 ps; and the leakage factor: 0 0.065 s^{-1}; and the NOE *R*-factor = 0.055.

single methyl internal correlation time (τ_{m3}) for the methyls on bound ligand and the enzyme. This makes the number of parameters to be optimized as six. We made the reasonable assumption[28] that the free ligand conformation is the same as the bound ligand conformation in the chicken liver DHFR complex. We also assumed that the protein conformation does not change between the free and complexed states.

STD NMR Measurements. The experimental STD data for the TMP/bovine DHFR complex were taken directly from Figure 2 of ref 20. We selected STD data sets at nine different saturation times (as shown in Figures 2 and 3). These saturation times (which include the STD-growth portions) were selected as the *S*/*N* ratio is expected to be significantly higher at these values. The STDs in Figure 2 of ref 20 were obtained at 298 K on a 600 MHz spectrometer. In the STD experiment the protein was irradiated with a Gaussian shaped pulse train at 0.9 ppm for saturated spectrum, and at −9.5 ppm for reference spectrum. The total recovery delay was set to 4 s. The NMR sample was prepared in D_2O with the ligand/protein ratio of 35:1 ([*E*] = 120 μM). STD spectra were collected with 16k data points to cover a sweep width of 12 ppm.

Since the chemical shift values of bovine DHFR are not available, we utilized the published assignments of human DHFR with bound methotrexate[29a] which is highly homologous (the residues in the binding pocket are identical except for R32). As the protein signals at 0.9 ppm were saturated in the STD experiment, for the computation of the Q-matrix in eq 1 we made the reasonable assumption that all protein protons with chemical shift values within 0.4 ppm of saturation frequency (0.9 ppm) were instantaneously saturated. These included the methyls of V8, A9, V10, L22, L27(D2), L67, I114, V115, V135, T136 as well as the β hydrogens of I7, L22(HB2), and I60. The chemical shift values of I7, I16, V50, and I51 methyl resonances were not listed in ref 29; however, the change in STD intensities by the inclusion of the saturation of these methyl resonances was found to be insignificant as these residues are not optimally located to produce large STDs.

3. Results and Discussions

The simulated annealing optimizations were performed by fitting the STD growth curves for the five different resonances in the TMP ligand at nine different saturation times. This resulted in a large data set with 45 STD intensities which were used as experimental constraints. The sampling range in the SA refinement for the τ_L is from 0.01 to 1ns, for the τ_P is from 0.1 to 100 ns, for the τ_m is 0.5 to 10 ps, and for the leakage relaxation is 0.05 to 0.3 s^{-1}. The optimized NOE *R*-factor is 0.076, and the optimized values for the parameters are τ_L: 0.101 ns; τ_P: 20.43 ns; τ_{m1}: 0.81 ps; τ_{m2}: 3.04 ps; τ_{m3}: 3.26 ps; and the leakage factor: 0.065 s^{-1}.

The STD buildup curves observed for the six-parameter optimization condition are shown in Figure 2. The low *R*-factor of 0.076 and the reasonable values of all the optimized parameters from the wide sampling range reveal the excellent quality of the optimization. The utilization of a large data set (containing 45 STD values) that contains STDs during their growth phase places more stringent requirements on the optimizations to be successful. This very low NOE *R*-factor suggests that the solution conformation of TMP/bovine DHFR complex is essentially identical to the crystal structure of TMP/chicken liver DHFR complex.

It is, however, interesting that a careful examination of the STD fits in Figure 2 suggests that the 7-CH_2 geminal protons

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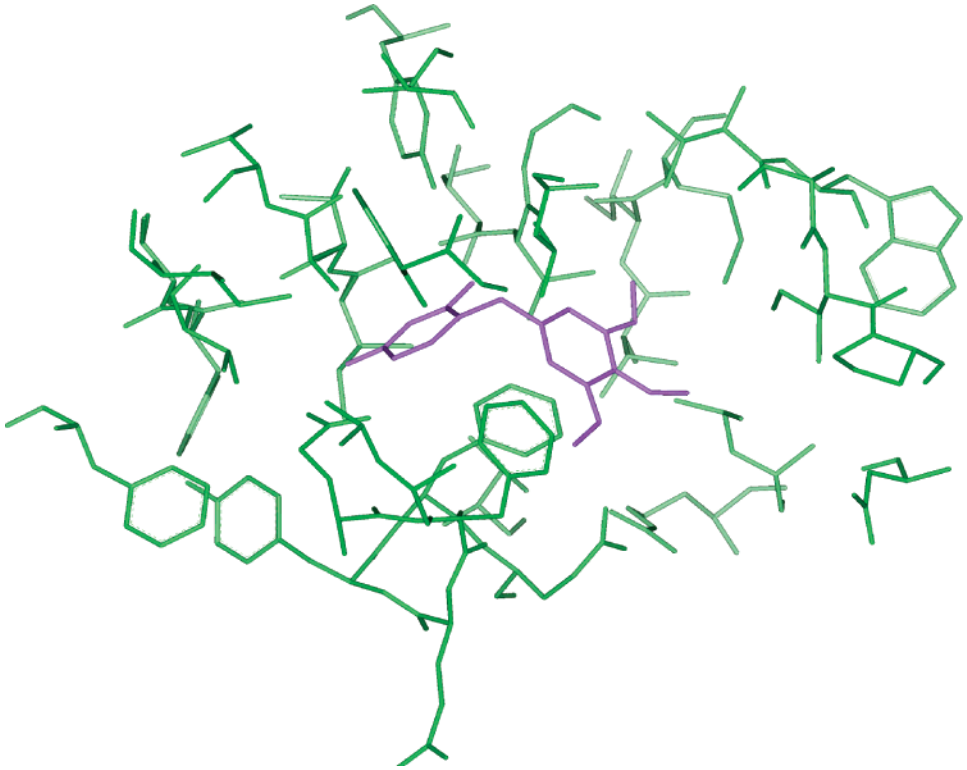

Figure 4. CORCEMA-ST optimized structure of bovine DHFR/TMP complex. (Green) Protein residues within the binding pocket included in the CORCEMA-ST calculations. (Purple) Ligand. The hydrogens are omitted for clarity.

and 4′OMe methyl signal show some systematic deviations from the experimental STD values. For 7-CH_2 geminal protons the predicted STD values are uniformly higher than the experimental values for all the saturation times. For the 4′OMe, the agreement is good for the first three saturation times, but after that the predicted values are lower than experimental values. When we carefully examined the bovine DHFR/TMP model, we found that the 7-CH_2 geminal protons can experience direct saturation from the nearest neighbor L22 methyl groups ($<$3Å). The chemical shift values of the L22 methyl groups are 0.51 and 0.8 ppm. In the STD experiment, the protein is irradiated at 0.9 ppm; thus, the L22 methyl at 0.51 ppm may escape saturation. A close examination of the bovine DHFR/TMP model reveals that L22 D1 methyl may experience high-field shift from the proximal aromatic rings, and thus, it may be the one to escape saturation in the STD NMR experiment. Thus, we performed an optimization by excluding L22 D1 methyl group from saturation. We simultaneously optimized six parameters and two torsion angles, θ_1 (C4−C5−C7−C1′) and θ_2 (C5−C7−C1′-C2′) of the TMP ligand. This optimization yielded an even lower NOE *R*-factor 0.055 with the following optimized parameters: τ_L: 0.082 ns; τ_P: 20.6 ns; τ_{m1}: 0.5 ps; τ_{m2}: 3.96 ps; τ_{m3}: 4.04 ps; the leakage factor: 0.065 s^{-1}; θ_1: −80.46° and θ_2 = 99.99°. The resulting STD buildup curves for all the five resonances are shown in Figure 3 and show a significant improvement in the fits for the 7-CH_2 and 4′OMe STDs. The optimized protein correlation time is reasonable for a protein the size of bovine DHFR.[30] The new torsion angles (θ_1 = −80.46° and θ_2 = 99.99°) are very close to the crystal structure values (θ_1 = −85.43° and θ_2 = 110.12°) of chicken liver DHFR complex. The resulting TMP/bovine DHFR complex is shown in Figure 4. The CORCEMA-ST calculations on the initial bovine DHFR/TMP model (based on chicken liver complex) already yielded a very low NOE *R*-factor of 0.076, and this is further improved (NOE *R*-factor = 0.055) with a very slight refinement of two torsion angles (θ_1 and θ_2 torsion angles of TMP ligand changed by ∼5° and ∼10°, respectively).

It is noteworthy that the C7 methylene protons show substantially smaller STDs compared to those of the remaining protons on the ligand. One might be tempted, using a simple "qualitative epitope mapping" based on STD intensities, to conclude that the C7 methylene protons are farther away from the protein than the remaining protons on the ligand. However, our quantitative epitope analysis presented here shows that the C7 methylene group is in fact in close contact with the protein. Its smaller STDs are a consequence of the stronger dipolar relaxation of the methylene protons. The significance of the variations in the relaxation rates of ligand protons on the magnitudes of the STD values has been originally pointed out by us previously.[10,11] In particular, we predicted that the geminal protons on a ligand are likely to show smaller STDs despite their close proximity to the protein.[10]

The results of CORCEMA-ST analyses and SICO optimizations are only as good as the quality of the experimental STD data being analyzed. The low *R*-factor we obtained confirms that the conformation we determined is highly compatible with the experimental STD data. This is reflected in the excellent agreement between the experimental and predicted STD intensities at several saturation times (Figure 3). It also reflects on the high quality of the experimental data in ref 20. Additionally, the resonances of TMP are well resolved, and thus there are no errors in the STDs from resonance overlap. The values of

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R-factors at global minima depend on a number of factors such as the signal-to-noise ratio of the STD-NMR spectra, ligand-to-protein ratio, errors in STD estimation due to resonance overlap, relative contribution of direct and indirect effects, and the number of parameters to be optimized. Our current work suggests that, when working with STD data sets with very high signal-to-noise ratio, no errors due to resonance overlap, and reasonably low ligand-to-protein ratio (which increases the magnitudes of STDs), and two torsion angles to be optimized, low *R*-factors of 0.1 or less are good target values to aim for in CORCEMA-ST refinements. Contrary to what is sometimes suggested in the literature, we show here that the STDs involving methyl resonances can also be analyzed quantitatively.

In this work we have utilized the crystal structure of the highly homologous chicken liver DHFR/TMP complex to serve as the starting model for the bovine DHFR/TMP complex and demonstrated that the crystal structure is essentially maintained in solution also. We wish to emphasize that the primary purpose of using the crystallographic structure was to provide an acceptable orientation of the ligand within the protein binding pocket rather than to have a starting conformation for the ligand close to the global minimum conformation since we had already demonstrated previously that our SICO procedure is able to successfully locate the global minimum conformation for *any arbitrary starting conformation* of the ligand[11] that is properly oriented in the binding pocket. In practice, however, the crystal structure for a homologous complex may not always be available. In such instances, as demonstrated recently in an elegant study,[31] one needs to utilize a robust computer docking program (such as AutoDock, FlexiDock, etc.) to generate several plausible binding modes, and test each one using CORCEMA-ST procedure to identify the mode that gives the best fit with the solution STD data, as judged by an acceptable NOE *R*-factor (vide supra). If necessary, the bound ligand structure in this binding mode can be subjected to a further torsion angle refinement using the SICO procedure to improve the NOE *R*-factor and thus identify the global minimum conformation. Unsatisfactory fits even after extensive optimizations may mean that the binding mode under consideration is not optimal, and alternate binding modes may need to be considered.

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4. Conclusions

In this work we have reported the solution structure of TMP within the binding pocket of bovine DHFR (Figure 4) using a novel protocol (SICO). The CORCEMA-ST analysis of STD intensities shows that the solution structure of bovine DHFR/TMP is essentially identical with the crystal structure of the chicken liver DHFR/TMP complex. Additionally, the excellent agreement between the experimental and predicted STDs for the TMP/DHFR complex also serves as a validation of the CORCEMA-ST methodology. As illustrated in this work, this method is readily applicable when the crystal structure data for a related complex is available. In addition, the combined use of robust computer docking programs[10,11,31] to generate various binding modes, high quality experimental STD-NMR data, CORCEMA-ST predictions, and SICO optimizations permits the determination of the bound ligand conformations in the binding pockets of target proteins even when crystallographic structures for related complexes are not available. Thus, the CORCEMA-ST method is likely to be useful in structure-based drug-design.

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