Probing Conformational Changes in Human DNA Topoisomerase IIα by Pulsed Alkylation Mass Spectrometry

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Background: The catalytic cycle of Top2 has multiple steps of reactions with corresponding conformational changes. We can monitor conformational dynamics of hsTop2α upon binding with cofactors and the inhibitor ICRF-193. Coordinated movements of the N-gate and DNA gate of hsTop2α occur with binding to cofactors and an inhibitor. Structural dynamics of hsTop2α can be detected during key catalytic steps.

Type II topoisomerases are essential enzymes for solving DNA topological problems by passing one segment of DNA duplex through a transient double-strand break in a second segment. The reaction requires the enzyme to precisely control DNA cleavage and gate opening coupled with ATP hydrolysis. Using pulsed alkylation mass spectrometry, we were able to monitor the solvent accessibilities around 13 cysteines distributed throughout human topoisomerase IIα by measuring the thiol reactivities with monobromobimane. Most of the measured reactivities are in accordance with the predicted ones based on a homology structural model generated from available crystal structures. However, these results reveal new information for both the residues not covered in the structural model and potential differences between the modeled and solution holoenzyme structures. Furthermore, on the basis of the reactivity changes of several cysteines located at the N-gate and DNA gate, we could monitor the movement of topoisomerase II in the presence of cofactors and detect differences in the DNA gate between two closed clamp enzyme conformations locked by either 5′-adenylyl β,γ-imidodiphosphate or the anticancer drug ICRF-193.

During replication and transcription, DNA can encounter several topological obstacles, such as supercoiling, catenation, and knotting (1–3). Type II DNA topoisomerases are enzymes capable of solving these DNA problems, and they play an essential role in separating catenated daughter chromosomes during DNA replication (4), which makes them critical targets for antibiotics and anticancer drugs.

Type II topoisomerases have a 2-fold symmetrical architecture forming a homodimer in eukaryotes and a heterotetramer (A2B2) in prokaryotes. A key step in the catalytic cycle of the topoisomerase II reaction is to pass a DNA segment through a transient double-strand break. In such a strand passage step, a segment of DNA serving as a gate (G-segment) is cleaved to create a staggered double-strand break with a 4-nucleotide protrusion at the 5′-ends. Each 5′-end of the DNA break is covalently linked to a catalytic tyrosine to form a transient DNA gate, which will be religated after the passage of the transport segment (T-segment) (5–7). By repeating the cycle of this reaction, type II topoisomerases are capable of reducing/introducing supercoils, decatenating/catenating DNA, or knotting/unknotting DNA.

Based on the structural information, type II topoisomerases can be divided into three major domains: the ATPase domain, cleavage/religation domain, and C-terminal domain. Each domain can be further divided into several subdomains. In the ATPase domain, GHKL (gyrase, histidine kinase, and MutL) and transducer domains form an ATP-binding pocket, and upon the binding of ATP, the GHKL domain can dimerize to form the N-terminal gate (N-gate) (8).

In the cleavage/religation domain, the winged helix domain and the adjacent tower domains are responsible for binding and bending the G-segment DNA (8). The TOPRIM (topoisomerase and primase) domain, connected to the winged helix domain through a flexible linker, can provide additional anchoring of the G-segment in the presence of a divalent cation, and the catalytic tyrosine at the winged helix domain coordinates with a second divalent cation to facilitate the DNA cleavage (9). The sequence of the C-terminal domain is not conserved. This domain has a more flexible structure and is more sensitive to proteases (10, 11).

Because the enzyme goes through a set of coordinated conformational changes to affect the strand passage reaction, our ability to probe these conformational states is critical for our understanding of the mechanistic basis of topoisomerase II reactions. We were thus interested in developing a facile method to monitor the structural dynamics of the enzyme during the catalytic cycle. Chemical protein footprinting methodologies have been developed to detect protein conformational changes utilizing reagents to selectively label the e-amine group of lysine (12, 13), the guanidino group of arginine (14, 15), or the thiol group of cysteine (16–19). Labeled proteins were then cleaved and separated by gel electrophoresis to examine the
alterations in cleavage patterns under specific conditions (12–14, 16, 17). More recent studies combined protein modification with mass spectrometry to study protein dynamics and conformational changes at a higher resolution (15, 18–20). For instance, cysteine modification followed by mass spectrometry has been applied to study the folding/unfolding of luciferase (18) and ligand binding with tubulin (19). In this study, we used pulsed alkylation of cysteines followed by mass spectrometric analysis to examine the protein dynamics of human topoisomerase IIα (hsTop2α).4 We determined that the reactivities of most of the 13 cysteines in hsTop2α are highly correlated with the accessibilities predicted from a structural model of hsTop2α with a few interesting exceptions. Comparing the reactivities of cysteines under conditions with or without cofactors and the topoisomerase inhibitor, we were able to further utilize this approach to detect conformational changes in hsTop2α, including flexible regions not shown in the Top2 crystal structures.

EXPERIMENTAL PROCEDURES

Enzyme Preparation—Recombinant hsTop2α was created by replacing the first 28 amino acids of hsTop2α with the first five amino acids of Saccharomyces cerevisiae Top2 (21), and the N terminus was tagged with a heart muscle kinase phosphorylation site and hexahistidin tag. The heart muscle kinase motif was added to potentially allow for end labeling of the protein for detection in an alternative gel-based cysteine footprinting method. The heart muscle kinase site contains the PKA consensus sequence (RRASV) (22). The C terminus was truncated (amino acids 1405–1530) to remove an intrinsic PKA consensus sequence (RKPST). To investigate the effect of the remaining C-terminal domain on the solvent accessibility, we designed a construct to remove as much of the C-terminal domain as possible with minimal perturbation of enzyme activities. Earlier work with Drosophila Top2 showed that removal of 240 residues does not affect the *in vitro* activities (23). Using homology as a guide, we generated a construct with a truncation of 310 residues (hsTop2α-CΔ310). This truncation also falls in between two previously described constructs in removing either 287 or 356 residues, with the former retaining full activities and the latter having somewhat reduced activities (24, 25). Both hsTop2α and hsTop2α-CΔ310 were overexpressed in yeast strain BCY123 and purified by Ni²⁺ affinity (nickel-nitriiotoriacetic acid resin, Qiagen) and ion exchange (POROS® HS column, Applied Biosystems) chromatography as described previously (26). Both enzyme constructs displayed activities comparable with those of the wild-type protein.

Sample Analysis of Liquid Chromatography/Electrospray Ionization Mass Spectrometry (LC/ESI-MS)—Tryptic peptides were analyzed by reverse-phase LC coupled with ESI-MS. A Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps, and an SCL-10A system controller) was coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (ABI/MDS SCIEX, Foster City, CA) equipped with an electrospray source. The LC system was operated at a flow rate of 200 μL/min with a linear gradient as follows: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 60% mobile phase B over 18 min and then increased to 100% mobile phase B over 5 min. Mobile phase A consisted of 98:2 (v/v) water/acetonitrile with 0.1% acetic acid. Mobile phase B consisted of 90:10 (v/v) acetonitrile/water with 0.1% acetic acid. A Zorbax C8 column (SB-C8, 2.1 mm (inner diameter) × 50 mm (5 μm), Agilent Technology) was used for LC/MS analysis with an injection volume of 10 μL. The mass spectra were acquired in the positive mode in the range of m/z 200–2000. The acquired spectra were then reprocessed using LC/MS reconstruct software (Analyzer QS software with the BioAnalyzer extension) to obtain the integrated peak area.

Sample Preparation for Evaluation of the Quantitative Labeling of Monobromobimane (mBrB)—Two batches of 210 μg of hsTop2α were individually prepared in 700 μL of TNE buffer (10 mM Tris-HCl (pH 7.9), 50 mM NaCl, and 0.1 mM EDTA). One batch was incubated with 50 mM mBrB (Calbiochem), whereas the other was labeled with 50 mM mBrB-d₆ (hexadeuterated mBrB). mBrB-d₆ was synthesized by the Duke University Small Molecule Synthesis Facility using a procedure developed previously (27). After a 10-min incubation at 37 °C, the samples were further labeled under denaturing conditions with 6 mM guanidine hydrochloride for 2 h at 37 °C. After completion of the labeling, the mBrB- and mBrB-d₆-treated samples were mixed in seven different ratios (5:0, 4:1, 3:2, 1:1, 2:3, 1:4, and 0:5). To remove excess labeling reagents (mBrB or mBrB-d₆), the samples were dialyzed with 4 × 1 liter of 100 mM NH₄HCO₃ solution with 5 mM 2-mercaptoethanol for 2 h at 4 °C. Dialysis was followed by trypsin digestion overnight at 37 °C, lyophilization, and resuspension with 50 μL of dH₂O before LC/ESI-MS analysis.

Procedure of Pulsed Alkylation Mass Spectrometry—100-μL samples of 30 μg of hsTop2α in TNE buffer were preincubated at 37 °C for 5 min. Samples were then pulsed with 0.5 mM mBrB-d₆ for 1 min and immediately quenched with 5 mM 2-mercaptoethanol for 1 min at 37 °C. After pulse labeling, the samples were incubated with the second reagent, mBrB (50 mM), for 10 min at 37 °C. The second labeling process was completed by denaturing the samples with 6 mM guanidine hydrochloride for 2 h at 37 °C. The samples were then processed as described above before LC/ESI-MS analysis. When specified, Mg²⁺, ATP, or AMPNP and ICRF-193 were included in the preincubation step. Each pulsed alkylation experiment was repeated at least three times.

Homology Model and Accessibility Analysis—A homology model of hsTop2α was constructed using the I-TASSER protein structure prediction server (28, 29). The sequence of hsTop2α, starting from residue 400, was submitted to the I-TASSER server. The crystal structure of the S. cerevisiae Top2 DNA-binding and cleavage domains (Protein Data Bank code 1BJT (30)) was specified as a template to produce a homology model that could be dimerized more easily. The crystal structure of the ATPase domain of hsTop2α (Protein Data Bank code 1ZXM (31)) was manually docked into its approximate location on the I-TASSER homology model using the KING visualization software program (32).

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4 The abbreviations used are: hsTop2α, Homo sapiens topoisomerase IIα; LC/ESI-MS, liquid chromatography/electrospray ionization mass spectrometry; mBrB, monobromobimane; AMPNP, S'-adenyllyl β, γ-imidodiphosphate.
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The solvent accessibility of the cysteines in our constructed model of hsTop2α was calculated using Probe all-atom contact analysis software (33). This software allowed us to roll a 1.4-Å radius ball around the model to calculate a visual dot representation of the solvent-accessible surface for the whole model. Then, for each cysteine in the model, similar dot representations were calculated, excluding all other residues. The overlap between the surface for the whole model and the surfaces for each cysteine’s thiol group gives an approximation of the solvent accessibility of each cysteine. This solvent accessibility was calculated by dividing the number of dots for a particular cysteine’s thiol in the whole model surface by the number of dots for that thiol if that cysteine was alone.

DNA Topoisomerase II Assay—The activities of hsTop2α and hsTop2α-CD310t were measured by unknotting and supercoil relaxation assays described previously by Hsieh (34).

RESULTS

Various reactions during the catalytic cycle of hsTop2α are tightly coupled with the dynamic protein structures. In an effort to monitor the conformational changes in hsTop2α, specific labeling of amino acid side chains offers an opportunity to capture the structural dynamics of the protein. This approach depends on labeling amino acids that have reactive side chains, such as the nucleophilic thiol of cysteine. Because hsTop2α has 13 cysteines in each monomer of the homodimer, evenly distributed throughout different domains, we applied pulsed alkylation combined with mass spectrometric analysis to hsTop2α to study its dynamics in response to cofactors and inhibitors. The procedure of cysteine-specific labeling in combination with LC/ESI-MS analysis is shown schematically in Fig. 1.

Labeling Reagents and Calibration Curve—Several alkylation reagents, including N-ethylmaleimide, iodoacetamide, and mBrB, were first tested in our experiments to label cysteines. N-ethylmaleimide failed to label reproducibly within the pulse time. Although iodoacetamide and mBrB both gave qualitatively similar results, we found that mBrB is a better reagent to distinguish between buried and exposed cysteines because it is sufficiently bulky to selectively label only surface cysteines within the pulse time. We also took advantage of its non-exchangeable hydrogens to synthesize a hexadeuterated derivative (mBrB-d6). This pair of labeling reagents should have similar chemical properties and equal labeling efficiency but distinguishable m/z signals, allowing us to perform a quantitative measurement by LC/MS. Thus, the results in this study are shown mainly with mBrB.

We first examined whether cysteine-containing peptides modified with either mBrB or mBrB-d6 could be detected by LC/MS and whether the intensities of the peptide signals were linearly proportional to their concentrations. After trypsin digestion, we were able to detect all cysteine-containing peptides by LC/MS (Table I). 11 of the 13 cysteines are located in different peptides, and only one pair of cysteines (Cys-997/Cys-1008) are in the same peptide. As a test for the linearity of quantification in labeling, cysteines were completely labeled with either mBrB or mBrB-d6, and mixed in various ratios. The signals of the cysteine-modified peptides could be identified and quantified by the integrated area of the monoisotopic peaks; Cys-170 is shown as an example in Fig. 2A. The reactivity of cysteine in pulsed alkylation experiments was determined by dividing the signal from the mBrB-d6-labeled peptides by the total signal from the peptides labeled with both reagents: reactivity = mBrB-d6/(mBrB-d6 + mBrB-h6). When the experimental ratios were plotted against the theoretical ratios (predetermined mixing ratios), calibration curves were obtained (Fig. 2B). The linearity of the calibration curves reveals that the ratios of the intensities closely reflect their relative concentrations. These data thus suggest that we can use a pair of
TABLE 1

Amino acid sequences and major ions of mBrB-\textsubscript{\textit{h}} or mBrB-\textsubscript{\textit{d}}-labeled tryptic peptides of \textit{hsTop2\textalpha}.

| Cysteinyl residue | hsTop2\textalpha amino acid sequence | Mass for mBrB-\textsubscript{\textit{h}} adduction | Mass for mBrB-\textsubscript{\textit{d}} adduction |
|------------------|--------------------------------------|-----------------|-----------------|
|                  |                                      | Observed m/z    | Calculated m/z  | Observed m/z    | Calculated m/z  |
| Cys-104          | MSCIR (102–106) [M + 2H]\textsuperscript{2+} | 400.183 | 400.179 | 403.203 | 403.198 |
| Cys-170          | LCNIFSTK (169–176) [M + 2H]\textsuperscript{2+} | 558.276 | 558.278 | 561.299 | 561.297 |
| Cys-216          | AGEMELKPFNGEDYTCITFDLHSK (201–225) [M + 3H]\textsuperscript{3+} | 1008.451 | 1008.460 | 1010.458 | 1010.473 |
| Cys-300          | WEEVTLMSEK (297–306) [M + 2H]\textsuperscript{2+} | 708.314 | 708.317 | 711.333 | 711.336 |
| Cys-392          | SFGSTCQASEK (387–397) [M + 2H]\textsuperscript{2+} | 688.805 | 688.808 | 691.825 | 691.826 |
| Cys-405          | AAIGCQVEELNWVWNK (401–416) [M + 2H]\textsuperscript{2+} | 931.983 | 931.992 | 935.003 | 935.010 |
| Cys-427          | CSAVK (427–431) [M + H] \textsuperscript{+} | 697.321 | 697.334 | 703.364 | 703.371 |
| Cys-455          | NSTECTLILEGDSAK (451–466) [M + 2H]\textsuperscript{2+} | 936.426 | 936.435 | 939.437 | 939.453 |
| Cys-733          | VLFCTC (729–735) [M + 2H]\textsuperscript{2+} | 524.269 | 524.267 | 526.272 | 527.285 |
| Cys-862          | VEPEWSIPIPMVGLINGEAGITGWSCK (836–863) [M + 3H]\textsuperscript{3+} | 1088.197 | 1088.210 | 1090.204 | 1090.223 |
| Cys-997/Cys-1008 | LQSTLCTCNMSLFDHVGLK (991–1010) [M + 3H]\textsuperscript{3+} | 863.739 | 863.743 | 867.723 | 867.768 |
| Cys-1145         | DELCR (1142–1146) [M + H] \textsuperscript{+} | 825.345 | 825.356 | 831.375 | 831.394 |

Reactivities of Cysteines in hsTop2\textalpha—To ensure that the pulse labeling procedure did not significantly alter the enzyme structure, the DNA-unknotting activity of the pulse-labeled protein was measured. Compared with the untreated controls, the labeled proteins retained 30% of the unknotting activity, indicating that the 1-min labeling condition we used did not lead to significant unfolding of the protein (supplemental Fig. 1). This notion can be further tested by comparing the cysteine reactivities with those predicted based on structural information. We therefore tested the cysteine reactivities without any cofactors as a way to first establish the base line of labeling efficiency. Under the same 1-min labeling conditions, we carried out a pulsed alkylation experiment with hsTop2\textalpha in the absence of Mg\textsuperscript{2+} and ATP for mass spectrometric analysis. Our results show that these 13 cysteines can be separated into three categories based on reactivity with mBrB-\textsubscript{\textit{d}} (Fig. 3A): 1) high reactivity (>10%), Cys-104, Cys-216, Cys-427, and Cys-1145; 2) medium reactivity (2–10%), Cys-170, Cys-300, Cys-392, and Cys-405; and 3) low reactivity (<2%), Cys-455, Cys-733, Cys-862, and Cys-997/Cys-1008. The reactivity experiment was also conducted with iodoacetamide and its deuterated derivative (idoacetamide-\textsubscript{d_3}), and the results show that these cysteines can be divided into three similar categories (supplemental Fig. 2). However, the quantitative differentiation among these cysteines is more distinct for the reactions with mBrB. To investigate if the reactivity of each cysteine is related to its solvent accessibility, we constructed a human Top2\textalpha homology model from the partial structures of yeast Top2 and human Top2\textalpha and compared our experimental results with the predicted cysteine accessibilities as analyzed by Probe software (Figs. 3B and 4A) (33). By using a water-sized probe to scan around the thiol groups of cysteines in the hsTop2\textalpha homology model, contact dots representing the level of solvent accessibility could be generated. Counting the contact dots of each cysteine allowed us to differentiate two groups of cysteines, buried and exposed (Fig. 4, B and C, respectively). It is interesting that the accessibility as calculated by probing the modeled structure has no intermediate reactivities. It is plausible that these intermediate reactivities are caused by breathing or other protein dynamics not accounted for in the static models. The accessibilities of Cys-405 and Cys-427 were excluded in our computational analysis because Cys-427 is absent in all Top2 crystal structures, and Cys-405, which is located at the interface of two docked struc-
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A

Cysteine Reactivity

![Cysteine Reactivity Diagram](image)

B

Computational Accessibility

![Computational Accessibility Diagram](image)

FIGURE 3. Measured and calculated cysteine accessibilities in hsTop2α. A, using pulsed alkylation with mass spectrometric analysis, 13 cysteines can be separated into three categories based on reactivity with mBrB-d₄: 1) high reactivity (≥10%), 2) medium reactivity (2–10%), and 3) low reactivity (<2%). B, based on the information from the homology model, the accessibilities of all cysteines were predicted by Probe all-atom contact analysis software. The cysteines can be categorized as exposed (>10%) or buried (<10%).

Cys-997/Cys-1008) are potential candidates for monitoring the conformational dynamics in the TOPRIM, winged helix, and Tower domains. Based on the homology model, the positions of Cys-455, Cys-733, and Cys-862 in hsTop2α are predicted to be covered inside the rigid secondary structures, preventing labeling by mBrB-d₄. The reactivity of Cys-997/Cys-1008 is minimal, suggesting that they are tightly shielded from the solvent. However, this is not consistent with the predicted accessibility in the structure, which shows that the side chain of Cys-1008 points outward and is exposed to the solution (Fig. 3B). Because this pair of cysteines is located close to the C terminus of the enzyme, which is not included in any crystal structures available, it remains possible that the presence of the C-terminal structure may mask their accessibility. Thus, this approach allows us to easily detect the accessibilities of cysteines in the entire protein, including the flexible regions and those absent in the crystal structures. Furthermore, it also provides a possibility to explore the conformational dynamics around these cysteines.

hsTop2α ATP Gate Conformational Change in the Presence of Mg²⁺ and AMPPNP—To examine the ATP gate movement, the reactivities of cysteines in hsTop2α were measured in the presence of 10 mM Mg²⁺ only, 1 mM AMPPNP only, or both. Compared with the control (absence of Mg²⁺ and AMPPNP), no obvious conformational changes were detected in the presence of either Mg²⁺ or AMPPNP (supplemental Fig. 3). However, with both present, there was a significant conformational change in the ATP gate region (Fig. 5A), and a similar result was also obtained with a different pair of labeling reagents, mBrB and iodoacetamide (supplemental Fig. 4 and supplemental Fig. 6). This change noticeably altered the reactivities of two groups of cysteines: 1) decrease in Cys-170, Cys-216, Cys-300, and Cys-392 and 2) increase in Cys-405 and Cys-455 (Fig. 5A). The reactivities of cysteines in the first group decreased by 2–3-fold. A possible explanation is that the GHKL and transducer domains in each of the pair of protomers move toward each other with Mg²⁺/AMPPNP, decreasing the accessibility of the cysteines in these two domains (Fig. 5B). Such a conformational change is consistent with a previous study that showed the ATP gate closes in the presence of Mg²⁺/AMPPNP (26, 35). However, this does not explain the decrease in reactivity for Cys-216, which is not in the location between the closing N-gate. Cys-216 is situated at the key position in the ATP-binding pocket. Its flanking residues, Thr-215 and Ile-217, interact with the liganded ATP by hydrogen bonding and van der Waals force, respectively (31). Thus, these interactions may constrain the flexibility of Cys-216, resulting in reduced reactivity.

Cys-405 and Cys-455 in the second group are located between the ATPase and cleavage domains. They displayed a reduced reactivity in the presence of AMPPNP, suggesting a coordinated conformational change in regions outside of ATPase domain upon N-gate closure. Cys-405, located at the bottom of the transducer domain, increased by 1.5-fold in reactivity, indicating that this location might be a hinge region for the movement associated with gate closure and thus may become more accessible to solvent (Fig. 5B). Cys-455 is located near the DNA gate (in the TOPRIM domain) and increased by 2-fold in reactivity upon AMPPNP binding, suggesting that...
AMPPNP-induced ATP gate closure causes a conformational change in the DNA gate that renders Cys-455 more solvent-accessible. As an interesting contrast, Cys-427, located in the flexible linker region between the TOPRIM and transducer domains, did not change in reactivity to mBrB-6 with Mg²⁺/H₁₁₀₀₁ and AMPPNP present. We also note that the reactivities of cysteines beyond the DNA gate remained unchanged, for example, Cys-1145 at the C-terminal coiled-coil domain. This result suggests that there is little or no conformational change in the C-terminal gate region after the binding of Mg²⁺/H₁₁₀₀₁ and AMPPNP.

Capturing the ICRF-193-linked ATP Gate Conformation—ICRF-193, a member of the family of bisdioxopiperazine drugs, is an antitumor agent that targets Top2 and forms a closed clamp protein complex by locking the N-gate in the ATPase domain (36). We measured the reactivities of cysteines in the closed clamp structure triggered by ICRF-193 binding. Although treatment with either ICRF-193 or Mg²⁺/AMPPNP can induce N-gate closure, we discovered that pulsed alkylation is capable of detecting differences between the ATP gate closure triggered by AMPPNP versus ICRF-193. With 150 μM ICRF-193, the reactivities of Cys-170, Cys-216, Cys-300, and Cys-392 had a similar decrease compared with AMPPNP-induced changes (Fig. 6). This similarity implies that the GHKL domain and part of the transducer domain are both required to move toward each other to close the ATP gate triggered by either agent. The reactivity of Cys-216 also remained at a similar level because nucleotides that restrict flexibility around Cys-216 were present under both conditions (AMPPNP and ATP). In contrast with the effect of AMPPNP, Cys-405 and Cys-455, near or at the DNA gate, did not significantly change in reactivity with ICRF-193. Therefore, the closed clamp complexes induced by AMPPNP and ICRF-193 could have different overall conformations. When Mg²⁺/AMPPNP triggers the closure of the ATP gate, the DNA gate likely adopts a more open conformation compared with that induced by ICRF-193. Cys-1145 at the C-terminal coiled-coil domain serves as a negative control whose reactivity is also unaffected by ICRF-193.

**DISCUSSION**

In this study, we have demonstrated that by using pulsed alkylation with mass spectrometric analysis, we were able to differentiate the levels of alkylating reactivities of cysteines in hsTop2α and probe the protein dynamics. There are two major determinants for the alkylation reactivity. One is chemical, with thiol nucleophilicity being influenced by its environment, and one is physical, with the reactivity being determined by the accessibility of a thiol group to solvent. Because there is a close correlation between the solvent accessibility calculated from a structural model and the measured reactivity, the accessibility factor is likely a critical one. However, the most useful information will be what can be extracted from the difference in reactivities under different reaction conditions, which can pinpoint the conformational changes occurring near a particular cysteine residue. We have shown here that we could detect the conformational changes in N-gate closure induced by AMPPNP and the anticancer drug ICRF-193. Furthermore, we also detected distinct conformational dif-
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Differences in closed clamp complexes resulting from the addition of either AMPPNP or ICRF-193, which were not observed previously upon comparing the crystal structures of the yeast ATPase domain bound to AMPPNP with or without ICRF-187 (37). In our experiments, both AMPPNP and ICRF-193 resulted in N-gate closure as indicated by the decreased reactivities of Cys-405 and Cys-455. The deduced movement of *hsTop2α* is shown diagrammatically in the model. The decreasing reactivities of Cys-170, Cys-300, and Cys-392 might result from the N-gate closing, and the increasing reactivities of Cys-405 and Cys-455 might be due to a change in the conformation near the DNA gate.

Besides monitoring the residues that are involved in conformational switches during N-gate closure, our analysis provided additional information that is not available in the crystal structures. Cys-405 is located near the C-terminal portion of the *hsTop2α* ATPase domain, and the structure of how it is connected to the cleavage/relation domain is unknown. Cys-427 is also missing in the crystal structures and is located in the linker region between the ATPase and cleavage domains. Its high accessibility is in accordance with the trypsin sensitivity in that domain (10). Both Cys-997 and Cys-1008 were found to have minimal thiol reactivities, whereas the structural model predicted that at least Cys-1008 should be more accessible (see Fig. 3, A and B). It is possible that the C-terminal domain missing in the crystal structure may mask Cys-1008. To test the hypothesis, we made a C-terminal 310-residue truncation construct (*hsTop2α*-C/H9004-310t), which retained the same catalytic activity as the full-length protein (data not shown). However, Cys-997 and Cys-1008 in *hsTop2α*-C/H9004-310t still had the minimal reactivities (supplemental Fig. 5). It is possible that the residues beyond the C-terminal 310 residues may be responsible for masking these residues or that there is a structural difference between the crystal and solution structures.

Protein side chain modification in combination with mass spectrometric analysis can be clearly applied to amino acids other than cysteine. However, previous studies demonstrated that *hsTop2α* is sensitive to thiol-reacting reagents and that cysteine modifications participate in a number of unique reactions (38–41). For instance, thimerosal, a mercury-containing compound that rapidly reacts with thiol groups, inhibited the topoisomerase II-decatenating activity (39). Thiopurines also inhibited topoisomerase II ATPase activity by alkylating thiol groups of cysteines (40). Both inhibitory effects can be abolished by adding reducing reagents, implicating cysteine modification in such reactions. However, it remains to be determined which cysteines are responsible for the inhibition. The mechanism of *hsTop2α* inhibition by quinone drugs is better characterized. Modification of Cys-392 and Cys-405 prevents the DNA relation step in the *hsTop2α* catalytic cycle, resulting in the accumulation of DNA breakage (41). In a recent study, the chemopreventive effect of dietary isothiocyanates was also shown to be related to cysteine modification of *hsTop2α*. In an *in vitro* assay, cysteines in the ATPase domain were found...
to be modified by benzyl isothiocyanate (42). The studies of cysteine modification of hsTop2α shed light not only on Top2 conformational dynamics and catalytic mechanism but also on the action of novel inhibitors that have potential application as antineoplastic agents.

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