Changes of Self-Association, Secondary Structure, and Biological Activity Properties of Topoisomerase II under Varying Salt Conditions†

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ABSTRACT: Topoisomerase II overexpressed in yeast was purified to near homogeneity. The milligram amounts of active enzyme obtained allowed its study by joint UV−circular dichroism, ultracentrifugation, and biological assays at different protein and salt conditions. First, sedimentation equilibrium was preferred over the other analytical ultracentifuge methods as it is based on firm theoretical grounds and does not require assumptions about the shape of the molecule. The tendency of topoisomerase II to self-associate into dimers was confirmed and shown to depend on both the enzyme concentration and the concentration of salt used. Analysis at five initial protein concentrations (from 0.08 to 1.05 mg/mL, *i.e.*, 0.5−65 μM) provided evidence for a single monomer−dimer equilibrium characterized at 150 mM KCl and 20 °C by an association constant, K_a, of ~4.8 10^5 M^{-1} and a $\Delta G°$ of ~−7.5 kcal mol^{-1}. Under these conditions, for a topoisomerase II concentration of 0.08 mg/mL (*i.e.*, 0.5 μM) in the ultracentrifuge cell, almost 80% of the enzyme were found dissociated. Increase of KCl (from 80 to 400 mM) in the medium provoked a continuous change of the association equilibrium so that a value of K_a ~10^5 M^{-1} corresponding to $\Delta G°$ ~−7 kcal mol^{-1} was found for topoisomerase II in 400 mM KCl at 20 °C. Second, circular dichroism (CD) showed the sensitivity of the topoisomerase II secondary structure to salt concentration, the observed variations being apparently dependent upon the ionic strength. For topoisomerase II in 150 mM KCl, *i.e.*, optimal salt conditions for the enzyme activity, the deconvolution of CD spectra revealed a secondary structure content of 40% α-helix and 30% β-turn that was the highest detected throughout the salt dependence experiments. Third, for topoisomerase II at varying KCl concentrations a remarkable correlation was observed between the data from CD spectroscopy (secondary structure) and catalytic activity. In contrast, although NaCl induced the same effects as KCl on the secondary structure of topoisomerase II, its influence on activity profile was found different, suggesting that K^+ and Na^+ exert distinct effects in the biological assays. Finally, the possible stabilization of topoisomerase II dimers induced by the interaction of the enzyme with its DNA sites is discussed.

In recent years there has been an upsurge of interest in the study of macromolecular associations because of the discovery of the dimeric or multimeric nature of many DNA-binding proteins. These include DNA topoisomerases which are involved in a number of vital processes such as replication and transcription (Dinardo *et al.*, 1984; Holm *et al.*, 1985; Vosberg, 1985; Wang, 1985; Maxwell & Gellert, 1986; Uemura & Yanagida, 1986; Brill *et al.*, 1987; Newport, 1987; Uemura *et al.*, 1987; Wang, 1987; Yanagida & Wang, 1987; Yang *et al.*, 1987; Zhang *et al.*, 1988). The main function of these enzymes is to catalyze changes in the topology of DNA by interconversion between topological isomers of DNA that requires the breaking and re-forming of phosphodiester bonds in the backbone (Tse *et al.*, 1980; Champoux, 1981; Caron & Wang, 1993).

DNA topoisomerase II, and its counterpart DNA topoisomerase I, are commonly found in prokaryotes and eukaryotes (Gellert, 1981; Liu, 1983; Wang, 1985; Maxwell & Gellert, 1986). Type II DNA topoisomerases have been extracted from many different mammalian species (Muller, 1985) including human sources (Pflugfelder *et al.*, 1988). The best studied type II DNA topoisomerase with respect to the physicochemical aspects is DNA gyrase (Gellert *et al.*, 1976). Complexes of gyrase with DNA have been analyzed by various techniques such as nuclease protection, filter binding, sedimentation, electron microscopy, and X-ray crystallography (Maxwell & Gellert, 1986, and references therein). Moreover, T4 topoisomerase II and eukaryotic topoisomerases II display similar properties, including the requirement for ATP in the relaxation of supercoiled DNA. However, these enzymes lack the supercoiling activity of gyrase.

The topoisomerase II gene (Top 2) of the yeast *Saccharomyces cerevisiae* has been cloned and found to be a single copy, essential gene, by Goto and Wang (1984). The overexpressed enzyme has been purified to near homogeneity in the same laboratory (Worland & Wang, 1989), and the various DNA reactions it catalyzes have been described. The sedimentation and gel filtration experiments with the purified enzyme have suggested that yeast topoisomerase II behaves as a dimer with two identical subunits (Goto *et al.*, 1984), a finding that is consistent with the homodimeric structure implicated for the enzyme of higher eukaryotes (Sander & Hsieh, 1983; Shelton *et al.*, 1983).

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We have combined ultracentrifugation analysis, far-UV−circular dichroism measurements, and catalytic assays to study DNA topoisomerase II from *S. cerevisiae*. This enzyme was obtained in milligram amounts in our laboratory through overexpression of yeast topoisomerase II (Worland & Wang, 1989). We confirm the ability of topoisomerase II to self-associate into dimers, although the enzyme demonstrates a single monomer−dimer equilibrium where the proportions of monomers and dimers strongly depend on both the protein concentration and salt concentration. It is thus assumed that, under enzyme assay conditions, only a minuscule amount of the topoisomerase II molecules is still dimeric and that the essential of dimer molecules are stabilized upon complexation with DNA. A good correlation between CD data affording the α-helix content in the enzyme and the catalytic activity data is observed for topoisomerase II in KCl solutions.

EXPERIMENTAL PROCEDURES

Enzyme Preparation. Yeast DNA topoisomerase II was obtained from *S. cerevisiae* as described (Worland & Wang, 1989). Briefly , DNA topoisomerase II was overexpressed in yeast from a multicopy expression plasmid kindly provided by James Wang (Harvard University) and purified by a four-step procedure consisting of yeast disruption (fraction I), elution from a polyethyleneimine/Celite column (fraction II), ammonium sulfate precipitation (fraction III), and phosphocellulose chromatography (fraction IV). Purified DNA topoisomerase II was dialyzed against the storage buffer adapted for spectroscopy methods (see Circular Dichroism). The purified enzyme preparation contained no detectable DNA topoisomerase I activity.

DNA Substrates. Supercoiled plasmid pBR322 DNA (>95% form I) was purchased from Sigma and used without further purification.

Enzyme Assay. Reactions were performed in standard buffer (10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$) containing 0.5 mM ATP and 150 ng of pBR322 DNA to which KCl was added independently from a stock solution to the indicated final concentrations. The reaction was initiated by the addition of DNA topoisomerase II and allowed to proceed at 30 °C for 10 min. Reactions were terminated by addition of 1% SDS, 0.05% bromophenol blue, and 10% sucrose, final concentrations. The samples were electrophoresed in 1% agarose gels at 2 V/cm for 18 h in Tris/borate/EDTA buffer at pH 8. Photographic negatives of the ethidium bromide stained agarose gels were scanned with a Joyce-Loebl Chromoscan 3 densitometer, and the peak areas of supercoiled DNA were determined.

Circular Dichroism. CD spectra were recorded with a Jobin-Yvon Mark IV high-sensitivity dichrograph connected to a PC microcomputer. Measurements were performed at 20 °C in quartz cells of 0.1 and 1 mm path length. Spectra were recorded in the far-ultraviolet region and expressed as mean residue molar ellipticity, $[\theta]$. For estimation of $[\theta]$, the molecular weight of DNA topoisomerase II was assumed equal to 160 000 (1429 amino acid residues) (Goto & Wang, 1984). The $[\theta]$ dimension is deg cm^2 $dmol^{-1}$ (or 1.745×10^{-5} rad m^2 mol^{-1}). For titrations, 180 μL of 4.2 μM protein solution in the spectroscopic buffer (10 mM Tris-HCl, pH 7.4, and 5 mM $MgCl_2$) was placed in the quartz cell, and small aliquots of concentrated KCl, NaCl, or $MgCl_2$ were added to adjust the salt concentration. In all experiments the total added volume of salt solution was below 10% of the initial volume, and dilution corrections are made. Secondary structures were estimated by processing CD spectra between 190 and 260 nm according to the method of Yang *et al.* (1986).

Ultracentrifugation Experiments. Experiments on DNA topoisomerase II were performed at 20 °C on a Beckman Model XLA analytical ultracentrifuge equipped with an AN-60-TI analytical rotor (Giebeler, 1992). Samples were prepared in a spectroscopic buffer consisting of 10 mM Tris−HCl, pH 7.4, and 5 mM $MgCl_2$, and loaded into 12 mm cells. We used standard aluminium filled Epon double sector cells with quartz windows.

(A) Sedimentation Equilibrium Analysis. The run duration was 72 h with a continuous radial scanning at 280 nm, with an average of 3 scans. Two separate experiments were performed.The first experiment consisted of examination of five different topoisomerase II concentrations (0.08, 0.2, 0.4, 0.8, and 1.05 mg/mL) at 150 mM KCl, at 7000 rpm. The second experiment consisted of analysis of the effects of varying KCl concentrations (80, 150, and 400 mM KCl) at 8500 rpm on a 0.4 mg/mL topoisomerase II solution. In the second experiment only 50 μL of FC43 fluorocarbon was placed in the bottom of each cell.

In the two experiments the concentration distributions were analyzed by fitting appropriate mathematical models using the XLA/EQASSOC analysis software supplied by Beckman. The EQASSOC program calculates the association constants and then permits the analysis of associating solutes containing monomers, dimers, trimers, and tetramers. The four parameters that are determined are M_1, the monomer molecular weight, and the logs of K_2, K_3, and K_4. The program utilizes either a subset of the data files or the primary files from the XLA instrument. A curve fitting algorithm is used to obtain results with the equilibrium equation:

$$C_r = C_1 + K_2(C_1)^2 + K_3(C_1)^3 + K_4(C_1)^4$$

$$C_1 = C_0 \exp[AM_1(r^2 - r_0^2)]$$

In these equations, C_r is the total concentration of reversible self-associating protein at any radial position, r, after attaining sedimentation equilibrium; C_1 is the monomer concentration; C_0 is the concentration at radial reference position r_0; and $A = [(1 - \bar{\nu}\varrho)\omega^2]/2RT$, where $\bar{\nu}$ is the partial specific volume, ϱ is the solution density, ω is the angular velocity, R is the gas constant, and T is the absolute temperature. The model is written for as many species as might appear to be necessary. Data were first analyzed according to a possible monomer−dimer, monomer−dimer−trimer and monomer−dimer−tetramer equilibrium. However, for the two highest association models, the highest association constant value was found to be at least 100-fold lower than the monomer−dimer association constant values. Morever, the latter values were found to be similar at the four different protein concentrations examined. It was thus concluded that the monomer−dimer association predominated over the other species in the selected experimental conditions, and we assumed a monomer−dimer equilibrium. A constrained value of 160 000 was used for the monomer topoisomerase II. The partial specific volume, $\bar{\nu}$, was taken as 0.73 cm^3 g^{-1}. This is intermediate between the two value

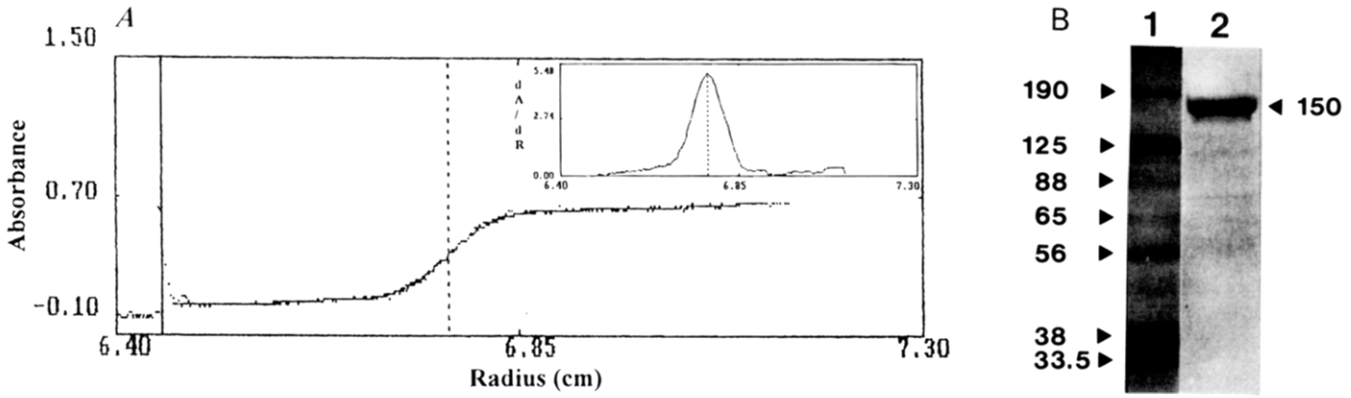

FIGURE 1: (A) Sedimentation velocity centrifugation of topoisomerase II. An ultraviolet photoelectric scanner trace showing that topoisomerase II migrates with a single symmetrical boundary. Path length is 12 mm; concentration = 0.8 mg/mL in 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, and 150 mM KCl, at 20 °C and 60 000 rpm. (B) SDS−polyacrylamide (7.5%) gel electrophoresis. Molecular weights in kDa are indicated on the left and right of the figure. Lane 1, marker lane: α_2-macroglobulin, β-galactosidase, fructose-6-phosphate kinase, pyruvate kinase, fumarase, lactic dehydrogenase, triosephosphate isomerase; lane 2, topoisomerase II.

0.7397 $cm^3\ g^{-1}$, calculated by the Wetlaufer procedure based on the content of Trp, Tyr, and Cys residues (Perkins, 1986), and 0.725 $cm^3\ g^{-1}$, obtained from the literature (Shelton *et al.*, 1983; Goto *et al.*,1984; Halligan *et al.*,1985). The mass extinction coefficient ($\epsilon = 0.9725$ mL $mg^{-1}\ cm^{-1}$) was also obtained from the Wetlaufer procedure. The solvent density, ϱ, value was taken as 1.003, 1.006, and 1.015 g cm^{-3} for 80, 150, and 400 mM KCl solutions, respectively.

(B) Sedimentation Velocity Analysis. Samples of 400 μL were centrifuged at 60 000 rpm, and radial scans of absorbance at 280 nm were taken at 8 min intervals throughout the 3 h duration of the experiment. The density of the solvent used in the experiments reduces the observed sedimentation coeficient by increasing the forces of buoyancy and frictional drag suffered by the topoisomerase II molecule. Thus, sedimentation coefficients are expressed in terms of a standard solvent, *i.e.*, water at 20 °C, so that:

$$s_{20,w} = s_{obs}[(1 - \bar{v}_b\varrho_{20,w})/(1 - \bar{v}_b\varrho_\tau)](\eta_\tau/\eta_{20})(\eta/\eta_0)$$

where ϱ_τ is the solvent density and $\varrho_{20,w}$ that of water at 20 °C, and η_τ/η_{20} is the relative viscosity of water at temperature T with respect to 20 °C and η/η_0 is the relative viscosity of the solvent with respect to water.

The computer program XLAVEL, supplied by Beckman, was used to analyze the data and determine sedimentation and diffusion coefficients. The sedimentation and diffusion method (s/D) is based on the relationship between the sedimentation coefficient and the molecular weight of the molecule. The well-known Svedberg equation is used for this calculation: $M = (RTs/D)(1 - \bar{v}\varrho)$.

Other Methods. Protein concentration was estimated either by optical absorption at 280 nm or by the method of Bradford (1976) with bovine serum albumin as the standard.

SDS−polyacrylamide gel electrophoresis was performed as described by Laemmli (1970).

RESULTS

Characterization. Large-scale preparation of overexpressed yeast topoisomerase II according to the procedure of Worland and Wang (1989) and Goto *et al.* (1984) is routinely made in our laboratory (Bojanowsky *et al.*, 1993). Such prepared topoisomerase II approaches homogeneity, as judged by SDS−polyacrylamide gel electrophoresis and sedimentation velocity (Figure 1, panels A and B). In the two cases topoisomerase II behaves as a single and monodisperse component. The protomer molecular weight is estimated to be 150 000 by SDS−polyacrylamide gel electrophoresis (Figure 1B) and does not differ from the one published by Goto *et al.* (1984).

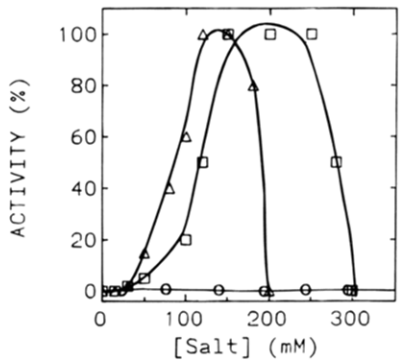

FIGURE 2: Effect of KCl (□), NaCl (△), and $MgCl_2$ (○) concentrations on the catalytic activity of topoisomerase II as measured by relaxation of supercoiled plasmid DNA. Relaxation assays were carried out as described under Experimental Procedures.

Activity Assay. The effect of the KCl, NaCl, and $MgCl_2$ concentration on the catalytic activity (as measured by relaxation of supercoiled plasmid DNA) of DNA topoisomerase II from *S. cerevisiae* resulted in the curves reported in Figure 2. The KCl curve was found to be bell-shaped, with the maximum activity observed between 100 and 250 mM, whereas the enzyme appeared inactive at KCl concentrations inferior to 50 mM and superior to 300 mM. Thus, these results confirmed the previous reports of Goto and Wang (1984). Remarkably, the NaCl curve differed from that obtained with KCl. It was still bell-shaped, but the profile of enzyme activity was found maximum only during a limited concentration range, of 120−150 mM. The difference of behavior displayed by the enzyme in the two monovalent salts, KCl and NaCl, suggested that cation fluxes influence the topoisomerase II activity as has been reported for anion fluxes in other cases (Mayes *et al.*, 1993). The influence of the divalent cation Mg^{2+} was also noteworthy in this respect, since $MgCl_2$, even at high concentrations, is unable to activate topoisomerase II in the absence of KCl. In the activity assays the enzyme demonstrated a requirement for small concentrations, 5 mM, of divalent $MgCl_2$ in addition

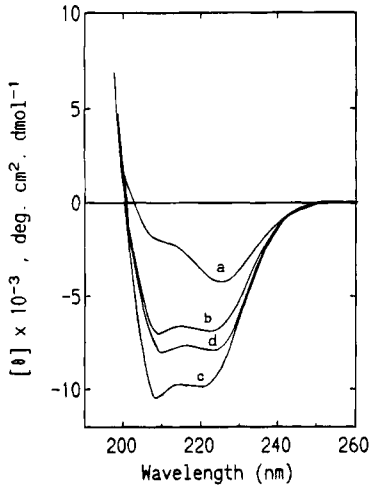

FIGURE 3: CD spectra measured in the peptide region for topoisomerase II as a function of KCl concentration in the spectroscopic buffer (10 mM Tris-HCl, pH 7.4, and 5 mM $MgCl_2$) at 20 °C. KCl concentrations: a, 50 mM; b, 80 mM; c, 150 mM; d, 400 mM. The spectra were recorded in a 1 mm cell.

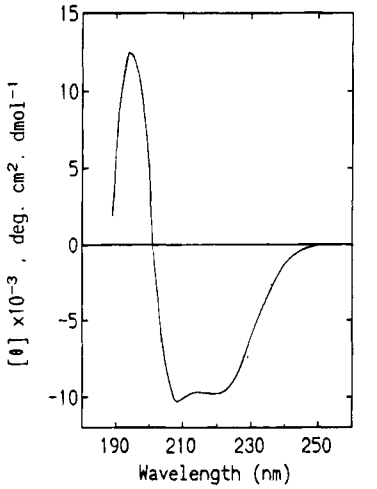

FIGURE 4: CD spectrum measured in the far-UV region for topoisomerase II in 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, and 150 mM KCl at 20 °C. The spectrum recorded in a 0.1 mm cell at a protein concentration of 4.2 μM, used to estimate the amount of secondary structures from the method of Yang *et al.* (1986).

to the 150 mM KCl, suggesting that Mg^{2+} acts as a cofactor of the reaction by binding to preferential cation binding sites either on topoisomerase II or on DNA (Osheroff, 1987).

Then, the question was whether these cations exert their influence through a specific conformational effect on topoisomerase II.

Circular Dichroism (CD). (A) Salt-Induced Conformation Transition. CD spectroscopy is applicable to solution samples that are difficult to obtain at high concentrations or to those having a high molecular weight, *i.e.*, nonamenable to methods such as NMR. These two features characterize the large and scarce DNA topoisomerase II molecule. By this CD analysis we aim to demonstrate that the secondary structure of DNA topoisomerase II is sensitive to salt effects and that conformational changes affect the enzyme activity. Thus, Figure 3 (curves a−d) shows the effects of increasing KCl concentration from 50 to 400 mM on the CD spectra of the protein solutions. Before going on, we may point out that within the range of salt concentration examined our topoisomerase II solutions did not display any turbidity, so that no problem of spectral distribution due to light scattering could emerge during our CD experiments. At low salt concentration (50 mM KCl) the CD spectrum of DNA topoisomerase II (Figure 3a) already displays features reminiscent of the α-helix structure with its characteristic two negative signals centred at ~226 nm ([θ] ~−4000) and ~205 nm ([θ] ~−2500). At higher KCl concentration (80 mM) (Figure 3b) the CD spectrum exhibits two well-defined negative bands at ~222 nm ([θ] ~−6000) and ~208 nm ([θ] ~−6500) demonstrating an increase of the α-helix content. The evolution of the CD signals continues with salt addition to become maximal for 100−150 mM KCl, where the spectrum is now typical of a secondary structure with a significant α-helix content ([θ]: ~−10 000 at 208 nm; −9000 at 222 nm) (Figure 3c). This picture persists up to ~250 mM KCl, and it is only for higher salt concentrations that we observe a substantial decrease of the CD intensities at 222 and 208 nm. At 400 mM KCl the change of the overall spectrum now characterizes an important loss of the α-helix content in the secondary structure of topoisomerase II (Figure 3d).

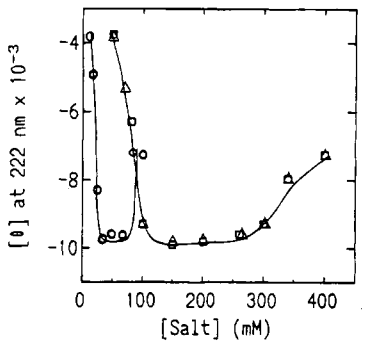

FIGURE 5: Conformational transitions of topoisomerase II induced by various salts in 10 mM Tris-HCl buffer, pH 7.4, and 5 mM $MgCl_2$ at 20 °C as measured by the change in ellipticity at 222 nm. The salts used were KCl (□), NaCl (△), and $MgCl_2$ (○).

(B) Secondary Structure Content. The protein secondary structure was predicted from the CD spectra by applying the method of Yang *et al.* (1986). The CD spectrum of a fresh topoisomerase II preparation in spectroscopic buffer (see Experimental Procedures) was recorded at 20 °C in the far-UV region between 190 and 260 nm at 150 mM KCl concentration (Figure 4), *i.e.*, the salt concentration where both the highest enzymatic activity and the largest negative CD signals are observed. The spectral deconvolution yielded the following contents: α-helix 39%, β-sheet 3.5%, β-turn 28%, and other 29%. These values were similar to those found for several well-known globular proteins (cytochrome *c*, lactate dehydrogenase, lysozyme, etc.) that have been investigated by X-ray diffraction and reviewed for their CD properties by Johnson (1992).

(C) Salt Type Effects. In order to detect the eventual conformational changes of the topoisomerase II molecule related to a salt specificity, we extented our CD experiments to include the analysis of NaCl and $MgCl_2$ effects. The salt dependence curves recorded by following [θ] at 222 nm for the three salts KCl, NaCl, and $MgCl_2$ are given in Figure 5. The curves for KCl and NaCl were found almost superimposable, and both indicated that maximum ellipticity, [θ] ~−10 000, was reached already at 100 mM of monovalent cation (K^+ and Na^+). In comparison, in the case of $MgCl_2$ the maximum of ellipticity, [θ] ~−10 000, was noted for

~40 mM salt concentration. This represents a divalent cation Mg^{2+} concentration that is approximately 3 times as low as the concentration of the monovalent cations K^+ and Na^+. It can be thus inferred from $I = {}^1/_2\Sigma[i]z_i^2$ (where $[i]$ and z_i are the concentration and the net charge of the ion i, and Σ implies a summation over all the ions in the solution) that the secondary structure of topoisomerase II is strongly mediated by the ionic strength. Also, addition of more salt to solution did not affect the CD ellipticity at 222 nm over a range of salt concentration that varies according to the valence of the cation examined. Thus, to produce a decrease of $[\theta]$ from −10 000 to −7500 ($\Delta[\theta]$ ~2500), only 60 mM divalent $MgCl_2$ was required relative to 400 mM monovalent KCl or NaCl.

Analytical Ultracentrifugation. (A) Sedimentation Equilibrium and Velocity Sedimentation. The sedimentation equilibrium method was applied to solutions of topoisomerase II in order to appreciate the reversible dissociation of the enzyme dimer and to evaluate the salt dependence of the monomer−dimer equilibrium. At sedimentation equilibrium the concentration distribution of two molecular species of different mass (monomer and dimer) will be identical if these molecular species are in rapid equilibrium (given the same mole ratio in each case) (Teller, 1973; Roark, 1976; Graceffa *et al.* 1988; Sackett & Lippoldt, 1991; Lechner, 1992). This is the preferred method for accurate studies of homogeneity and molecular associations. The sedimentation velocity method uses the speed with which a molecule moves toward the bottom of a cell (outermost boundary) to yield the sedimentation coefficient, s, related to the molecular weight (Svedberg & Pederson, 1940; Schachman, 1959; Cann *et al.*, 1994).

(B) Characterization of Monomer−Dimer Equilibrium. A good demonstration of reversible equilibrium may be obtained by centrifuging several different initial concentrations of a protein to equilibrium at a single rotor speed and obtaining the same equilibrium constant for all distributions. Solutions of topoisomerase II at five concentrations were centrifuged to equilibrium. In Figure 6 are presented the results for one of these experiments performed with topoisomerase II at 0.8 mg/mL in 150 mM KCl solution. The concentration distribution and the results of the fitting procedure are shown in panel A. Each data point is shown as a dot (~100 data points in this experiment), and a solid line represents the values predicted by the fit. The quality of fit to the data is clearly put in evidence. However, since the fit does not reflect systematic error, the difference between the fit values and data values are also given in panel B. We see that the values of residuals are small (root mean square deviation error ~0.004) and are approximately randomly distributed about zero.

Fits of the quality as obtained above are typical of all the data sets reported in Table 1for the five concentrations of topoisomerase II at 150 mM KCl. The fitting procedure yielded the same association constant, K_a, of ~5 × 10^5 M^{-1}, leading to a standard free energy change on association, $\Delta G°$ ~−7.6 kcal mol^{-1}, from $\Delta G° = -RT \ln K_a$. With this K_a value, according to the association of mass, at protein concentrations of 0.08, 0.2, 0.4, 0.8, and 1.05 mg/mL in cells, topoisomerase II was approximately 80%, 60%, 50%, 35%, and 30% dissociated, respectively. It is noteworthy that a unique K_a value predicts so well the concentration distribution with five different topoisomerase II concentrations. This

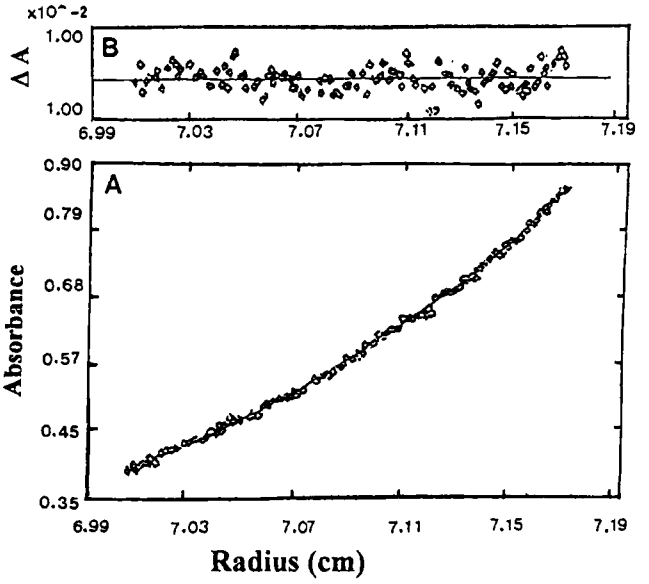

FIGURE 6: Distribution of concentration and residuals to the fit for topoisomerase II as a monomer−dimer equilibrium. Sedimentation was performed at 7000 rpm in an AN-60-TI analytical centrifuge. Topoisomerase II was used at a concentration 0.8 mg/mL in 10 mM Tris-HCl, pH 7.4 buffer containing 150 mM KCl and 5 mM $MgCl_2$. Panel A: Concentration distribution of topoisomerase II. Panel B: Distribution of the residuals from the fit to the monomer−dimer equilibrium model. The line shows the best fitting curve for the monomer−dimer equilibrium model.

Table 1: Effect of Protein Concentration on Topoisomerase II Dimer Formation at 20 °C, 150 mM KCl, Analyzed by Sedimentation Equilibrium

[protein] (mg/mL)	ln K_a	$10^5 K_a$ (M^{-1})	$10^{-6} K_d$ (M)	$\Delta G°$ (kcal mol^{-1})[a]
0.08	12.96	4.27	2.34	−7.03
0.2	13.15	5.14	1.94	−7.65
0.4	13.06	4.69	2.13	−7.6
0.8	13.13	5.04	1.98	−7.64
1.05	13.09	4.84	2.06	−7.62

[a] From: $\Delta G° = -RT \ln K_a$.

accounts for a single equilibrium between dimers and monomers of topoisomerase II at 150 mM KCl concentration (see also analysis of data in Experimental Procedures).

(C) Salt Dependence of the Molecular Equilibrium Shown by Sedimentation Equilibrium. Ionic (*i.e.*, charge-neutralizing) interactions are involved in entropic change on protein association (Ross & Subramanian, 1981). To learn about the salt influence on the monomer−dimer equilibrium, we examined the effect of salt concentrations on a 0.4 mg/mL protein solution. In addition to 150 mM KCl, we tested 80 and 400 mM KCl for the ability of these salt concentrations to alter the topoisomerase II activity in the various biological assays. The concentration distributions and the results of the fitting procedure are shown in Figure 7. The more representative values are given in Table 2. The values of the association constant, K_a, decrease progressively in increasing the salt concentration, from 7.5 × 10^5 M^{-1} at 80 mM KCl to 1 × 10^5 M^{-1} at 400 mM KCl, indicating that the topoisomerase II molecules dissociate parallel to the increase of the salt concentration. On the basis of these K_a, the dissociation of topoisomerase II in the cells corresponds to about 40% (80 mM KCl), 50% (150 mM KCl), and 75% (400 mM KCl). The topoisomerase II dissociation upon increase of salt concentration can also be illustrated by the

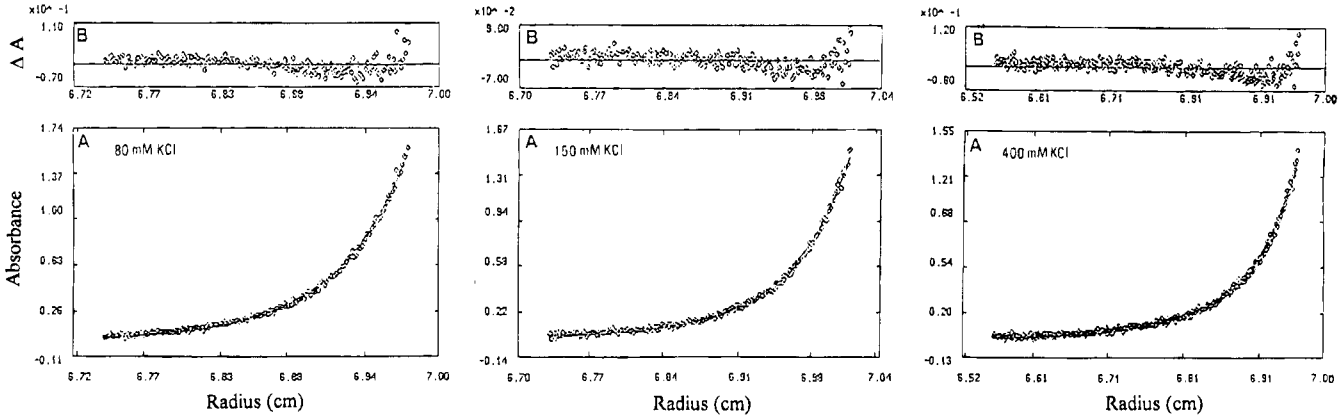

FIGURE 7: Concentration distributions (A) and residuals (B) to the fit for topoisomerase II as a monomer−dimer equilibrium. Sedimentation was performed at 8500 rpm in an AN-60-TI analytical centrifuge. Topoisomerase II was loaded at a concentration 0.4 mg/mL in 10 mM Tris-HCl, pH 7.4 buffer, with 5 mM $MgCl_2$, at three differents concentrations of KCl (80, 150, and 400 mM).

Table 2: Salt Effects on Topoisomerase II Molecular Equilibrium at 20 °C, 0.4 mg/mL Protein, Analyzed by Sedimentation Equilibrium and Sedimentation Velocity

[KCl] (mM)	ln K_a	$10^5 K_a$ (M^{-1})	$10^{-6} K_d$ (M)	$\Delta G°$ (kcal mol^{-1})[a]	sedimentation coeff ($s_{20,w}$)	mol wt
80	13.53	7.5	1.33	−8.01		268 000[b]
150	13.04	4.6	2.17	−7.72	8.9	230 000
						235 000[b]
400	11.51	1	10	−6.81	7.9	186 000
						190 000[b]

[a] See Table 1. [b] Calculated from sedimentation equilibrium by using the equation: $M = 2RT/(1 - \bar{\nu}\varrho)[d(\ln(c))/dr^2]$ (see Experimental Procedures).

reduction of the free energy association, ΔG°, from −8 kcal mol^{-1} at 80 mM KCl to −6.8 kcal mol^{-1} at 400 mM KCl, thus underlining the contribution of ionic interactions to the change of entropy upon association. The extent of variation suggests that the association of topoisomerase II is relatively high in salt sensitivity, although the ionic interactions presumably coexist with the hydrophobic interactions in stabilizing the dimers.

(D) Salt Dependence of the Molecular Weight Distribution Shown by Sedimentation Velocity. Estimates of the apparent molecular weight of topoisomerase II under varying salt concentrations were provided by sedimentation velocity experiments. The protein topoisomerase II in solution at a fixed concentration of 0.4 mg/mL was examined at two KCl concentrations, 150 and 400 mM, already used in sedimentation equilibrium experiments. The *s* values obtained from sedimentation velocity are presented in Table 2. These are consistent with a decrease of the topoisomerase II molecular weight due to the dimer dissociation induced by increase of KCl concentration, in conformity with the sedimentation equilibrium results.

DISCUSSION

Sedimentation equilibrium and sedimentation velocity experiments reveal the sensitivity of topoisomerase II self-association toward both the protein concentration and the salt concentration. The methods which are widely used for the investigation of protein association−dissociation had never been applied to topoisomerase II. Still, sedimentation equilibrium in the analytical centrifuge is the most powerful technique for the absolute determination of molecular weight averages and distributions of macromolecules (Van Holde, 1975; Lechner, 1992). It is based on firm theoretical grounds and allows great precision. The appearance of a new generation of centrifuges with associated computers and programs make the use of the technique easier and faster (Giebeler, 1992).

The present results demonstrate that topoisomerase II exists preferentially in a monomer−dimer equilibrium which is highly sensitive to both protein concentration and salt concentration. As indicated in the Experimental Procedures, inclusion of terms for higher order assemblies such as trimers and tetramers does not improve the fit. For instance, if significant amounts of tetramer were present, residuals would increase together with the increase of protein concentration. No such residuals variation was detected in the conditions of our experiments.

The existence of a dimeric topoisomerase II has been previously pointed out on the basis of ultracentrifugation in a gradient of either sucrose (Haligan *et al.*, 1985) or glycerol (Shelton *et al.*, 1983; Goto *et al.*, 1984). These experiments have failed, however, to demonstrate the existence of monomer−dimer equilibrium and hence the sensitivity of this equilibrium to salt concentration. Only Goto *et al.* (1984) found incremental differences in the sedimentation rates of *S. cerevisiae* topoisomerase II when gradients were run in 150 mM KCl and 1 M KCl. This is not surprising since the equilibrium position in favor of a polymeric state(s) is a function of sucrose concentration and can thus result in failure to detect the coexistence of monomer in the equilibrium (Cann *et al.*, 1994). For proteins undergoing reversible self-association, the molecular crowding effect of small solute may well displace the interaction in favor of the polymeric state, the extent of this change in equilibrium position being dependent upon the concentration of small inert solute (Shearwin & Winzor, 1988). The results presented here demonstrate that topoisomerase II dimers dissociate into monomers by increasing salt concentration as shown by the ca. 5-fold decrease of K_a from 150 mM KCl to 400 mM KCl. In contrast, the dimer is stabilized by decreasing the salt concentration from 150 to 80 mM KCl (K_a increases by almost twice in this interval). The reduction of ΔG° value observed from 150 to 400 mM KCl (the ionic stength, *I*, varies from ~0.2 to ~0.5) represents about 10%, and thus the contribution of ionic interactions to the entropy variation during topoisomerase II dimerization must be rather significant.

The contribution of ionic interactions to the conformational change of topoisomerase II molecules was then assessed by circular dichroism. CD measurements have been in use for over 20 years to analyze the secondary structure of protein, and the reliance on such studies has stemmed from several aspects of the method: ease and sensitivity, nondestructive nature, examination of conformations, and conformational changes in dilute solutions (Fasman, 1976; Johnson, 1992). Above all, the method is well adapted to the influence of medium modification, including salt effects. A question is to what extent the secondary structure within each subunit of the enzyme is sensitive to salt concentration? Then, is the change of secondary structure related to the variation of monomer−dimer populations? Finally, is the biological activity better correlated to changes observed on secondary structure or on monomer−dimer populations? The variation of $\Delta G°$ illustrates the interaction of K^+ ions with topoisomerase II and their competition with the protein association. Contacts of protomers through charged side chains can thus be considered as an important contribution to the stabilization of topoisomerase II dimers. However, the variation of CD signals observed in the salt dependence experiments provides a clear demonstration of a two-step change affecting the topoisomerase II secondary structure. This clearly contrasts with the continuous change observed within the monomer and dimer populations in the ultracentrifugation cells. In the first step, increase of KCl up to 150 mM promotes a stabilization of topoisomerase II secondary structure (α-helix) that correlates with dimer dissociation. In the second step, increase of KCl from 150 to 400 mM provokes a substantial destabilization of the secondary structure which correlates now with the continuing dimer dissociation. Thus, all this suggests that the conformation of topoisomerase II is shaped by a subtle network of interactions created within the secondary, tertiary, and quaternary structures and between these structures and salt ions. It is noteworthy that fluorescence anisotropy measurements still in progress in our laboratory confirm the salt effects on the solutions of topoisomerase II. Added to topoisomerase II in KCl solutions of 150 and 400 mM, the long-lived fluorescent probe pyrene (benzophenanthrene, $C_{10}H_{10}$) shows a decrease of anisotropy, indicating a marked variation of the rotational correlation time of the molecule. Taken together with the above results, these observations allow one to conclude on the existence of a conformational change in topoisomerase II occurring simultaneously to the dissociation of dimers through a KCl effect between 150 and 400 mM. However, to answer the question whether the secondary structure interferes directly in the association properties, and vice versa, remains a difficult task.

Ionic interactions are not necessarily the major noncovalent forces stabilizing the topoisomerase II association and secondary structure. Other interactions due to van der Waals contacts and especially hydrophobic forces are also relevant. Many proteins self-associate through the interactions of motifs containing several leucine or isoleucine or any hydrophobic residues, generally oriented on one face of a helical structure, together with supporting electrostatic interactions intercalated between the hydrophobic side chain interactions (O'Shea *et al.*, 1989, 1993). Interestingly, such a motif assimilated to a leucine zipper has been recently identified in the C-terminal part of topoisomerase II (Caron & Wang, 1993; unpublished results). This motif could be a good candidate for the autoassociation of the enzyme.

Finally, the bell-shaped curve obtained for the catalytic activity of topoisomerase II as a function of KCl concentration confirms the ones previously reported by Goto and Wang (1984) and Goto *et al.* (1984). It is remarkable that as far as KCl is concerned, the activity profile of topoisomerase II parallels the conformational changes visualized by CD experiments. Thus, the existence of a biologically active secondary structure taking place at a given dimer−monomer equilibrium and that is induced by 150 mM KCl is therefore pointed out for topoisomerase II. Other proteins also demonstrate such correlated changes (Yoo & Albani, 1990; Yoo & Lewis, 1992), although this cannot be generalized to all proteins (Shire *at al.*, 1992).

Yet, it turns out that a conformation such as the one stabilized by 150 mM KCl or NaCl is not the only requirement for biological activity of the enzyme. Especially, the difference in activity profiles observed between NaCl and KCl suggests a dependence of topoisomerase II activity on the identity of monovalent cation rather than a simple dependence on ionic strength. A possible explanation for this difference between Na^+ and K^+ is that K^+ produces its own stabilizing effects either directly on the target DNA (Pinnavaria *et al.*, 1978; Sundquist & Klug, 1989; Williamson *et al.*, 1989; Guschlbauer *et al.*, 1990; Sen & Gilbert, 1990) or on the DNA−topoisomerase II complex or the complex associated to cofactors (Liu *et al.*, 1983; Osheroff *et al.*, 1983; Sander & Hsieh, 1983; Pommier *et al.*, 1984; Osheroff, 1986, 1987).

To summarize, in present experimental conditions the topoisomerase II molecule displays a dimer−monomer equilibrium and a defined folded structure, the latter being unstable below and above a threshold KCl concentration. Obviously, according to the association constant here determined, it can be extrapolated that the dimer population will be very low at the nanomolar enzyme concentrations used for biological assays. Yet, it should be stressed that, by virtue of the fixation of the protein to DNA, dimerization domain would be constrained in terms of both location on binding sites and mutual orientation. Under such circumstances the midrange interactions ($K_a \sim 5 \times 10^5\ M^{-1}$) seen in free topoisomerase II could become very strong, and the combined effect of immobilization, *i.e.*, reduction of the unfavorable entropic contribution, and of adequate orientation could substantially increase the effective K_a. In contrast, a strongly stabilized pre-existing topoisomerase dimer could prevent fine adjustment of the protein on the DNA structure, the latter macromolecule generally displaying more rigidity than the protein during DNA−protein complexation (Spolar & Record, 1994). For comparison, it is remarkable that the (DNA) linker histone H5 (GH5) exhibits a self-association constant as low as $\sim 5 \times 10^3\ M^{-1}$ (Maman *et al.*, 1994). Such a weak K_a does not prevent the GH5 molecules from creating an axial core through polymerization in the chromatin fiber and contributing to stability of the formed complex. In a completely different example offered by membranes, it has been shown that a great reduction of the unfavorable entropy of immobilization and orientation of proteins in close proximity can increase the likelihood of dimer formation by ca. 10^6-fold (Grasberger *et al.*, 1986).

It is tempting to propose the following scheme of sequential events, also relevant for topoisomerase II at catalytic concentrations:

Inactive topoisomerase II	Active topoisomerase II	Inactive topoisomerase II
DIMER ⇔ monomer	dimer ⇔ monomer	dimer ⇔ MONOMER
"Unfolded" structure	"Folded" structure	"Unfolded" structure
does not recognize DNA	*recognizes DNA, dimer is concomitantly stabilized*	*does not recognize DNA*
80 mM KCl	150 mM KCl	400 mM KCl

To conclude, we hope that this work on the structural properties of yeast topoisomerase II may, in the future, aid the synthesis of a new class of anticancer drugs that are effective in preventing the enzyme self-association.

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