Leroy, J. L., Kochoyan, M., Huynh-Dinh, T., & Gueron, M. (1988) *J. Mol. Biol. 60*, 223-238.
Lindahl, T. (1982) *Annu. Rev. Biochem. 51*, 61-87.
Loeb, L. A., & Preston, D. B. (1987) *Annu. Rev. Genet. 20*, 201-230.
Manoharan, M., Ransom, S. C., Mazumder, A., Gerlt, J. A., Wilde, J. A., Withka, J. M., & Bolton, P. H. (1988a) *J. Am. Chem. Soc. 110*, 1620-1622.
Manoharan, M., Mazumder, A., Ransom, S. C., Gerlt, J. A., & Bolton, P. H. (1988b) *J. Am. Chem. Soc. 110*, 2690-2691.
Mazumder, A., Gerlt, J. A., Rabow, L., Absalon, M. J., Stubbe, J., & Bolton, P. H. (1989) *J. Am. Chem. Soc. 111*, 8029-8030.
Mazumder, A., Gerlt, J. A., Absalon, M. J., Stubbe, J., Cunningham, R. P., Withka, J. M., & Bolton, P. H. (1991) *Biochemistry 30*, 1119-1126.
Pardi, A., & Tinoco, I. (1982) *Biochemistry 21*, 4686-4693.
Pardi, A., Morden, K. M., Patel, D. J., & Tinoco, I. (1982) *Biochemistry 21*, 6567-6574.
Randall, S. K., Eritja, R., Kaplan, B. E., Petruska, J., & Goodman, M. F. (1987) *J. Biol. Chem. 262*, 6864-6870.
Sagher, D., & Strauss, B. (1983) *Biochemistry 22*, 4518-4526.
Schaaper, R. M., Kunkel, T. A., & Loeb, L. A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 487-491.
Serianni, A. S., Kline, P. C., & Snyder, J. R. (1990) *J. Am. Chem. Soc. 112*, 5886-5887.
Shaka, A. J., Keeler, J., & Freeman, R. (1983) *J. Magn. Reson. 53*, 313-340.
Shaka, A. J., Lee, C. J., & Pines, A. (1988) *J. Magn. Reson. 77*, 274-293.
States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286-292.
Swaminathan, S., Ravishanker, G., & Beveridge, D. L. (1991) *J. Am. Chem. Soc. 113*, 5027-5040.
Vesnaver, G., Chang, C., Eisenberg, M., Grollman, A. P., & Breslauer, K. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3614-3618.
Wemmer, D. E., & Reid, B. R. (1985) *Annu. Rev. Phys. Chem. 35*, 105-137.
Wilde, J. A., Bolton, P. H., Mazumber, A., Manoharan, M., & Gerlt, J. A. (1989) *J. Am. Chem. Soc. 111*, 1894-1896.
Withka, J. M., Swaminathan, S., Beveridge, D. L., & Bolton, P. H. (1991) *J. Am. Chem. Soc. 113*, 5041-5049.
Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, John Wiley & Sons, New York.

Mg^{2+}, Asp^-, and Glu^- Effects in the Processive and Distributive DNA Relaxation Catalyzed by a Eukaryotic Topoisomerase I†

P. Arsène Der Garabedian,* Gilles Mirambeau,‡ and Jacqueline J. Vermeersch
Laboratoire d'Enzymologie, Université Pierre et Marie Curie, URA 554 CNRS, 96 Boulevard Raspail, 75006 Paris, France
Received May 3, 1991; Revised Manuscript Received July 18, 1991

ABSTRACT: The salt requirement for the catalysis of DNA relaxation carried out by a eukaryotic DNA topoisomerase I from *Candida* was reexamined with plasmid pBR322 DNA. Two levels of analysis were considered: the initial velocity of the overall reaction and the mode of this reaction (processivity vs distributivity). When looking at the monovalent salts from the first level, the replacement of Cl^- by Glu^- or Asp^- greatly enhanced the salt range over which the enzyme was active. Moreover, the initial velocity reached an optimal value for a higher salt concentration in this case. For the cationic counterpart, K^+ was a little more effective than Na^+ and much more so than NH_4^+. Addition of 4 mM magnesium chloride affected both the range and the optimum of the initial velocity differentially, depending upon the monovalent salt, but with a general stimulating tendency. On the other hand, when the Mg^{2+} salt was varied, substitution of chloride by aspartate enhanced the optimum of the initial velocity for a fixed KCl concentration. In addition, magnesium aspartate ($MgAsp_2$) and magnesium glutamate ($MgGlu_2$) allowed the reaction to occur even without monovalent salt and over an extended range. Magnesium was also shown to directly interact with the general catalysis (K_d = 2.5 mM). From the second level of analysis, the presence of Mg^{2+} (except with NH_4Glu), the substitution of Cl^- by Glu^- or Asp^-, and a lower monovalent salt concentration than that used for the velocity optimum were required to promote the processive mode. The mode of action of magnesium as well as the interest of the substitution of the chloride ion by glutamate or aspartate are discussed. Finally, the data derived from both analyses are supported by the proposed existence of an intermediary complex between supercoiled DNA, Mg^{2+}, and DNA topoisomerase I, which may explain the processive relaxation of supercoils into DNA (presumably the predominant in vivo catalysis).

DNA topoisomerases (topo) are enzymes that alter the topological states of DNA. They can be divided in two classes (topo I and topo II), depending upon their mode of action, and are widely distributed in prokaryotes and eukaryotes. It is now well established that these enzymes play a crucial role in most of the biological processes involving DNA (Wang, 1985; Osheroff, 1989).

The eukaryotic type I topoisomerase has been studied extensively since its discovery in 1972 (Champoux & Dulbecco, 1972). Its prominent catalytic activity, i.e., DNA relaxation, can be summarized as follows: after binding to some preferential site on the DNA, topo I efficiently acts by creating a transient break in one strand and by promoting the passage

† This work was supported by grants from CNRS.
* To whom correspondence should be addressed.
‡ Present address: Unité de Physicochimie des Macromolécules Biologiques, Institut Pasteur, 75724 Paris Cedex 15, France.

0006-2960/91/0430-9940$02.50/0

of the intact strand through this break. One passage modifies the linking number (Lk) by a value of 1 (Maxwell & Gellert, 1986; Osheroff, 1989). When the DNA is supercoiled, the free energy contained in the superhelix enables as many passages as possible to fully relax the double helix regardless of whether the supercoils are negative or positive. Complete DNA relaxation can be accomplished by topo I via a processive or a distributive mechanism, depending at each catalytic step upon the enzyme's propensity to engage in another catalytic step or to dissociate from its DNA binding site (McConaughy et al., 1981; Pulleyblank & Ellison, 1982; Shuman & Prescott, 1990). The chemical reaction (i.e., double transesterification), which introduces the transient nick, and the different possible sequences of the DNA binding site where it occurs have been intensively studied during the last few years (Champoux, 1978; Been & Champoux, 1984; Bonven et al., 1985). More recently, the tyrosine involved in the chemical reaction has been mapped (Eng et al., 1989; Lynn et al., 1989). The effect of the DNA's topological properties on the relaxation behavior has been examined, especially at the DNA binding and transient nick levels (Muller, 1985; Camilloni et al., 1988, 1989). In contrast to the above studies, the catalytic aspect of the overall reaction has been poorly examined, presumably because of the difficulty in approaching it experimentally (Pulleyblank & Ellison, 1982; Wang, 1985; Caserta et al., 1990).

In addition to these biochemical studies of the eukaryotic topo I, several investigations have focused attention on the general involvement of this enzyme in rapidly eliminating the accumulation of topological stress in DNA by means of tracking processes, like replication or transcription (Liu & Wang, 1987, Brill & Sternglanz, 1988; Giaever & Wang, 1988; Wu et al., 1988). On the other hand, it has been shown that the nuclear DNA, the locus of the most extensive and quasipermanent transcription, retained topo I at high-affinity binding sites where relaxation seems to be highly efficient (Bonven et al., 1985; Muller et al., 1985; Busk et al., 1987). These results strongly emphasize the processive properties of such an enzyme and prompted us to closely reexamine which are the in vitro requirements that drive the enzyme to be processive (or distributive).

In vitro catalysis by eukaryotic DNA topo I is generally carried out in an electrolyte mixture containing NaCl or KCl alone at concentrations in the range of 0.03–0.27 M (Vosberg et al., 1975; Brun et al., 1981; Dynan et al., 1981; Pulleyblank & Ellison, 1982; Badaracco et al., 1983; Tricoli & Kowalski, 1983) and often in the presence of 4–10 mM $MgCl_2$ (Vosberg et al., 1975; Dynan et al., 1981; Liu & Miller, 1981; Goto & Wang, 1984; Thrash et al., 1985; Lazarus et al., 1987; Shaffer & Traktman, 1987; Uemura et al., 1987). In contrast to prokaryotic topo I, for which magnesium is essential for maximal activity, the eukaryotic enzyme is able to function in the complete absence of a divalent cation. However, type I topoisomerases from diverse eukaryotic sources are stimulated 2–25-fold by the presence of magnesium, calcium, or manganese ions (Osheroff, 1989). It is noted that most of the in vitro studies were made under distributive conditions. A classical way to shift from a processive to a distributive mode of catalysis and vice versa consists of changing the salt concentration and/or composition in the reaction buffer. It may then be expected that a reduction of the salt concentration will decrease the dissociation (K_d), favoring a processive reaction. This has been shown for the eukaryotic topo I when going from 200 mM NaCl (predominantly distributive) to 10 mM KCl plus 10 mM $MgCl_2$ (predominantly processive) (McConaughy et al., 1981; Shuman & Prescott, 1990).

In vitro, protein–DNA interactions depend strongly upon electrolyte activity: they behave thermodynamically like ion-exchange reactions in which the formation of a complex is accompanied by the release of a large number of electrolyte ions. Thus, the presence of salts in the reaction buffer will contribute not only to the activation of each macromolecule to adopt its adequate configuration but also to the association and dissociation rates of a given protein–DNA complex (Record et al., 1985). One purpose of choosing a mixed electrolyte in vitro is precisely to allow the formation of such a complex in an aqueous diluted solution; another is to stimulate the intracellular ionic environment. Furthermore, general macromolecular interactions are much more salt-dependent in an aqueous solution than in the intracellular medium (Leirmo et al., 1987). Among several explanations of this dependency comes one from the discrimination made by a cell between cationic and anionic counterparts present in the external medium. In the case of *Escherichia coli*, for example, external potassium can easily penetrate into the cytoplasm up to a high concentration, when external chloride is kept outside and is physiologically compensated by biosynthesis or activation of endogenous weaker anions, notably glutamate (Richey et al., 1987). Therefore, the anionic composition inside a test tube must be carefully considered. The relative effects of glutamate and chloride have already been compared in several in vitro protein–DNA interactions and in the corresponding catalysis when the protein involved was an enzyme (Leirmo et al., 1987; Zwelling et al., 1988; Griep & McHenry, 1989). In general, it appears that glutamate favors complex association and slows its dissociation, thereby extending the salt range over which a protein–DNA complex is stable.

Because the stability of the topo I–DNA complex is an important feature during DNA relaxation, we evaluated the ability of glutamate, aspartate (another cellular concentrated dicarboxylic anion), or chloride to promote relaxation in different combinations with monovalent (Na^+, K^+, NH_4^+) and divalent (Mg^{2+}) cations, using pBR322 as the DNA substrate and the eukaryotic topo I isolated from the imperfect fungus *Candida guilliermondii* var. *membranaefaciens*. We determined, for each salt combination, the initial velocity and the processive or distributive behavior of the corresponding reaction as a function of the concentration. A comparison of this newly described enzyme isolated from *Candida* with commercially available topo I both from wheat germ (Promega) and calf thymus (BRL) gave the same results (not shown).

Materials and Methods

Cell Cultures. The yeast *C. guilliermondii* var. *membranaefaciens* was cultured as previously described (Der Garabedian & Vermeersch, 1987).

Purification of Topoisomerase I. Unless otherwise specified, the purification was carried out at 0–4 °C.

(*A*) *Cell Lysis.* About 40 g (wet weight) of *Candida* cells was suspended in a buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM KCl, 1 mM EDTA,[1] and 1 mM DTT (TKED) plus 0.1 M sucrose and lysed in a Dyno Mill disrupter (Willy A. Bachofen AG Maschinenfabrik, Basel, Switzerland) at 3000 rpm for 5 min. The lysate was removed from the cell disrupter and washed with 400 mL of TKED + 0.1 M sucrose. Lysate and wash fluid were centrifuged at 4300*g* for 20 min. The

[1] Abbreviations: DTT, dithiothreitol; KAsp, potassium aspartate; NaAsp, sodium aspartate; $MgAsp_2$, aspartate hemimagnesium; KGlu, potassium glutamate; NaGlu, sodium glutamate; $MgGlu_2$, magnesium glutamate; PEG, poly(ethylene glycol); LDS, lithium dodecyl sulfate; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

supernatant was set aside, and the pellet was resuspended in 100 mL of TKED + 0.1 M sucrose and then centrifuged at 4300*g* for 20 min. This process was repeated once more with the remaining pellet, and the three supernatants were pooled (fraction I).

(*B*) *Polymin P and Ammonium Sulfate Fractionations.* To fraction I, 5% polymin P, pH 7.4, was added slowly to a final concentration of 0.7%. The suspension was stirred for 30 min and centrifuged at 8500*g* for 30 min, and the supernatant was set aside (fraction II). Solid ammonium sulfate was added to fraction II to 50% saturation (O °C) and the mixture was stirred for 60 min. The precipitate was removed by centrifugation (30 min at 8500*g*). Additional solid ammonium sulfate was added to the supernatant to 80% saturation, the mixture was stirred for 60 min, and the precipitate was collected by centrifugation (30 min at 45000*g*).

The pellet was dissolved in 30 mL of TKED + 0.1 M sucrose and was loaded at 50 mL/h onto a GF05 Trisacryl column (2.6 × 31 cm) equilibrated and eluted with TKED + 0.1 M sucrose to remove the ammonium sulfate (fraction III).

(*C*) *Batchwise CM Trisacryl Fractionation.* CM Trisacryl (6 mL packed volume) equilibrated with 50-fold-diluted TKED + 0.1 M sucrose was added to fraction III and stirred at 4 °C for 1 h. The resin, recovered by centrifugation for 5 min at 12000*g*, was added to 7 mL of TED (TKED without KCl) + 0.1 M sucrose + 0.1 M KCl, stirred for 15 min, and then centrifuged for 5 min at 12000*g*; the supernatant was set aside and the process was repeated once more with TED + 0.1 M sucrose + 0.1 M KCl (a), twice with TED + 0.1 M sucrose + 0.15 M KCl (b), twice with TED + 0.1 M sucrose + 0.2 M KCl (c), twice with TED + 0.1 M sucrose + 0.25 M KCl (d), and twice with TED + 0.1 M sucrose + 1 M KCl (e). The active fractions (b and c) were pooled (fraction IV) and the process was repeated with NaCl instead of KCl: 0.025, 0.1, 0.15, 0.2, and 1 M. The active fraction eluted at 0.15 M (fraction V).

Protein Determination. The protein concentration was determined by using the method of Schaffner and Weissman (1973) with bovine serum albumin as the standard. Protein samples were analyzed on a 5–20% polyacrylamide gradient slab gel in the presence of SDS as described by Laemmli (1970) and silver stained by the simplified ultrasensitive technique of Oakley et al. (1980) in order to determine molecular mass. The presence of topo I antigen was confirmed by protein blotting (Oddou et al., 1988).

DNA Substrate. Negatively supercoiled pBR322 DNA was prepared as previously described (Clewel & Helinsky, 1970).

Enzyme Assay. Incubation buffer generally contained, in 20 μL, 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT, 0.25 μg of negatively supercoiled DNA (pBR322), and 0.8 ng (1 unit) of topo I from *Candida* (fraction V), and 25–400 mM KCl, KGlu, KAsp, NaCl, NaGlu, NaAsp, NH_4Cl, or NH_4Glu, and/or 0–60 mM $MgCl_2$ or aspartate hemimagnesium ($MgAsp_2$). The mixture was incubated for various times at 37 °C. The enzymatic reactions were then stopped by addition of 0.1 volume of 4% LDS, 0.3% bromophenol blue, and 50% sucrose. Topoisomerization products were separated by horizontal agarose gel electrophoresis (Keller, 1975) as described below.

The protein purification has been followed by using standard assay conditions: 4 mM $MgCl_2$ and 150 mM KCl with an incubation time of 5 min.

Agarose Gel Electrophoresis. The electrophoretic analysis was performed in 1.2% agarose slab gels (11.5 × 13.5 × 0.5 cm) in an electrophoresis buffer (pH 7.8) containing 36 mM Tris, 1 mM EDTA, and 30 mM sodium phosphate. Current was applied at 50 V for 4 h, with recirculation of buffer between reservoirs. Following electrophoresis, gels were stained for 20 min in ethidium bromide at 2 μg/mL in bidistilled water and then transferred into bidistilled water for another 20 min. Gels were then illuminated from below with a shortwave ultraviolet light plate and photographs were taken with Pola Pan 4 × 5 in. instant film. Densitometric scanning of photographic negatives enabled the activity to be quantified as the disappearance of supercoiled substrate (pBR322).

Results

Enzyme Properties. The DNA topo I that we have partially purified (446-fold) from *Candida* comprises a polypeptide with a molecular mass of about 100 kDa, as revealed by its electrophoretic mobility on SDS–polyacrylamide gels followed by protein blotting with an anti-calf thymus topo I serum. Currently, the molecular mass of the type I enzyme purified from the yeast is thought to be in the range of 90–135 kDa (Badaracco et al., 1983; Goto and Wang, 1984; Thrash et al., 1985). The *Candida* topo I is capable of relaxing both negatively and positively supercoiled DNA in the presence of monovalent and divalent cations, a typical property of eukaryotic topoisomerases (Champoux & Dulbecco, 1972).

In order to discern similarities or differences in the properties of various eukaryotic type I topoisomerases, we compared certain physiological and biochemical characteristics of three of these enzymes (from *Candida*, wheat germ, and calf thymus). They remained equally active at pHs 7.8–8.0 and over a broad range of temperatures (20–41 °C) at any molar amounts of monovalent and divalent ions (not shown), which is similar to previously published results. For the subsequent studies, we considered *Candida* topo I as a paradigmatic enzyme to study the catalysis of DNA relaxation, but we systematically checked that calf thymus and wheat germ topo I led to the same results.

Reaction Kinetics. Most investigations of topoisomerases have not been systematically carried out according to classical enzyme protocols: for example, most relaxation reactions were performed for 10 or 30 min without any consideration of the reaction kinetics. As the analysis of the enzyme properties is much too limited under these conditions, we decided to control systematically the kinetics of our incubations: this enabled us to measure the initial velocity and to distinguish between processivity and distributivity.

Figure 1 shows the time course and initial velocities of DNA relaxation, i.e., the complete relaxation of DNA, at different enzyme concentrations and a fixed initial supercoiled DNA concentration in a standard salt medium (4 mM $MgCl_2$ and 150 mM KCl). Kinetics were determined by scanning a negative film of an agarose gel under appropriate conditions (see Materials and Methods). These results show that, up to 2.5 min, the overall relaxation rate of the supercoiled DNA was directly proportional to both the time (initial velocity) and the enzyme concentration from 0.4 to 1.9 units (excess DNA). On the other hand, the complete relaxation of supercoiled DNA catalyzed by the *Candida* topo I followed classical pseudo-first-order kinetics, as previously shown for the avian enzyme (Pulleyblank & Ellison, 1982; Caserta et al., 1990). For simplicity, we defined one unit as the mean enzyme concentration value and 2.5 min as the mean time and compared all experiments at their initial velocity. This rate was calculated from the best-fit line of the plot of the concentration of relaxed DNA versus time.

In our assay, one topo I unit (0.8 ng of protein) gave a protein:DNA ratio of 0.003 (w/w). This value indicates a

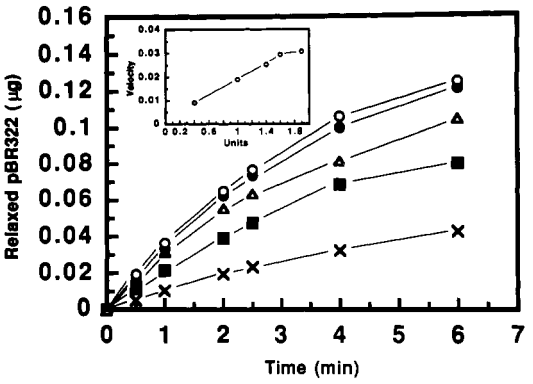


FIGURE 1: Enzyme assay: DNA relaxation as a function of time at different concentrations of *Candida* topo I. Enzyme concentrations (units): ×, 0.4; ■, 1.0; △, 1.4; ●, 1.6; ○, 1.9. Initial supercoiled DNA is kept constant (0.13 μg/20 μL). One enzyme unit is defined as the enzyme concentration that relaxes 0.02 μg of DNA (pBR322) in 1 min at 37 °C. Inset: Initial velocity (calculated as [relaxed DNA]/t) as a function of the enzyme concentration. Incubation time = 2.5 min.

5-fold excess of DNA molecules compared to protein, which indeed signifies a very high excess of available topo I binding sites. This is supported by earlier studies, which had shown a range of 200–500 sites on a DNA molecule containing about 5000 bp (Maxwell & Gellert, 1986). Under such conditions, we may postulate that our *Candida* topo I preparation catalyzed DNA relaxation to a significant extent. Therefore, the salt effects on this catalysis that are presented in the following sections must directly affect the enzyme's properties. Moreover, this hypothesis can be extended to eukaryotic topo I in general, as the same results were obtained with calf thymus and wheat germ topo I (not shown). Unless otherwise specified, all the data presented below were obtained at initial velocities with pBR322 DNA as the substrate.

Effects of Monovalent Salts on the Overall Relaxation Kinetics. Figure 2 illustrates the effects on DNA relaxation velocity of several combinations of three monovalent cations (K^+, Na^+, and NH_4^+) and three monovalent anions (Cl^-, Glu^-, and Asp^-) with or without a divalent cation (Mg^{2+} from a $MgCl_2$ salt). Marked differences were observed with the various combinations.

Mixing a fixed $MgCl_2$ concentration (4 mM) with different monovalent salts gave different results. With KCl, KAsp, and NaAsp, optimal relaxation velocities were the same either with or without $MgCl_2$. In contrast, in the presence of $MgCl_2$, a small stimulation appeared for NaGlu, NH_4Glu, NaCl, and NH_4Cl and a considerable one for KGlu. Furthermore, a 5-fold stimulation factor was observed between 200 mM NaCl on one hand and 100 mM KCl plus 4 mM $MgCl_2$ on the other hand. This is consistent with the classical effect already mentioned for the magnesium in the DNA relaxation catalyzed by a eukaryotic topo I.

For the three monovalent chloride salts, different optimal concentrations were noted in the presence and in the absence of divalent cation Mg^{2+}. On the contrary, the replacement of Cl^- by the dicarboxylic anions Glu^- or Asp^- did not shift the salt concentration required for maximal velocity.

The rate of the reaction reached an optimum value of 0.04 μg of relaxed DNA formed/min for 200–250 mM KAsp (with or without 4 mM $MgCl_2$) and for 200–250 mM KGlu with 4 mM $MgCl_2$. NH_4Glu and KGlu, both without $MgCl_2$, had slower velocities across the entire range of concentrations studied. The optimal concentration was always 100–150 mM for the monovalent chloride salts but reached 200–250 mM for the Asp and Glu salts.

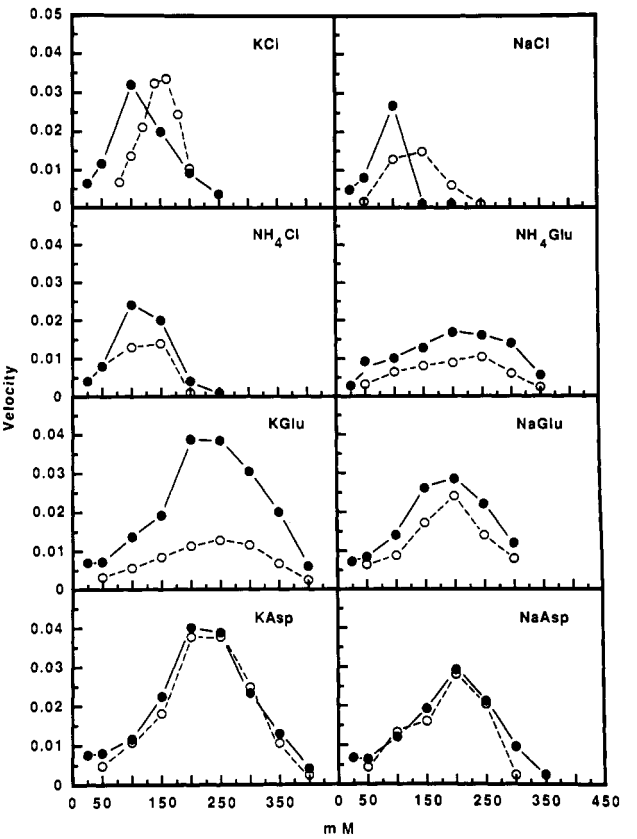


FIGURE 2: Effect of the monovalent anion concentration with or without $MgCl_2$ on the rate and extent of *Candida* topo I relaxation: determination of the optimum salt concentration. Incubation time = 2.5 min at 37 °C. ●, with 4 mM $MgCl_2$; ○, without $MgCl_2$. Velocity = micrograms of relaxed DNA formed per minute.

The concentration of monovalent salts that enabled total DNA relaxation was demonstrable for the chloride salts up to 150 mM for Na^+ in the presence of $MgCl_2$; 250 mM for Na^+, without $MgCl_2$, and for NH_4^+, with or without $MgCl_2$; and 250 mM for K^+, with or without $MgCl_2$. Activity persisted up to 400 mM for KGlu and KAsp and up to 350 mM for NaGlu, NaAsp, and NH_4Glu with or without $MgCl_2$. Similar downshifts in both the NaCl range and optimum due to the presence of a divalent cation were previously reported for the type I topoisomerase from rat liver (McConaughy et al., 1981) and for vaccina virus DNA topo I (Shuman et al., 1988). The relative effects of KGlu and KCl were quite similar to those already shown by others on the enzymatic activities of *E. coli* RNA polymerase and various restriction enzymes (Leirmo et al., 1987), *E. coli* DNA polymerase III (Griep & McHenry, 1989), topo II from *Drosophila* (Zwelling et al., 1988), and *E. coli* Rep helicase (Lohman et al., 1989) as well as on *E. coli* SSB protein binding to DNA (Overman et al., 1988).

Effects of Monovalent Salts on the Catalytic Behavior of DNA Relaxation. It is possible to discriminate visually between the distributive and processive pathways that lead to complete DNA relaxation. If a large amount of intermediate topoisomers appear during the course of this reaction, it tends to be distributive. In contrast, when the DNA is directly relaxed, the reaction tends to be processive throughout. Both types of catalysis are illustrated in the gel electrophoresis photos in Figure 3.

The conditioning of the reaction by the different combinations used in Figure 2 is shown in Figure 4. In the absence of $MgCl_2$, predominantly distributive catalysis occurred with all monovalent salts (except NH_4Glu) and at all concentrations

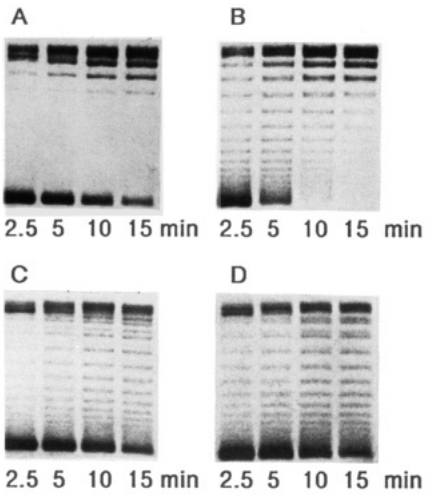


FIGURE 3: Electrophoretic patterns of processive (A) and distributive (B–D) relaxation kinetics catalyzed by *Candida* topo I, either with or without divalent cation. (A) 150 mM KGlu + 4 mM $MgCl_2$; (B) 300 mM KGlu + 4 mM $MgCl_2$; (C) 150 mM KGlu; (D) 300 mM KGlu. Incubation times 2.5, 5, 10, and 15 min at 37 °C.

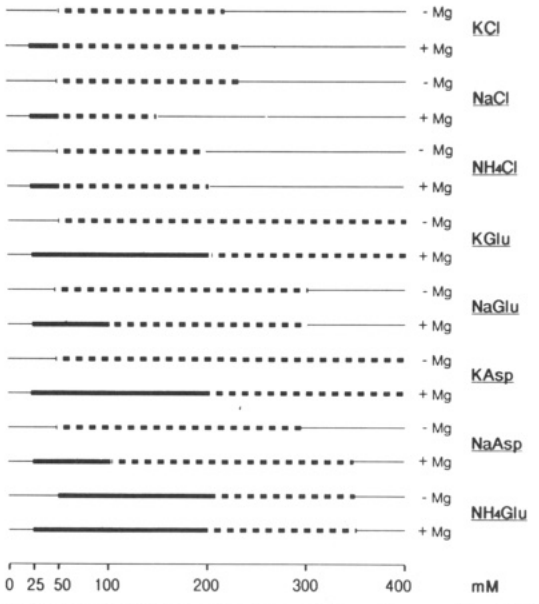


FIGURE 4: Effect of monovalent ions either with or without $MgCl_2$ on the processivity and distributivity of *Candida* DNA topo I. Incubation time = 2.5 min at 37 °C. Bold line, processive relaxation; broken bold line, distributive relaxation. Light lines represent no relaxation activity.

studied. In the presence of 4 mM $MgCl_2$, the topoisomerase reaction proceeded in a processive manner at lower concentrations of K^+ and Na^+ associated with Cl^- (25–50 mM), whereas at higher concentrations, the enzyme reaction became increasingly distributive. This phenomenon was observed previously with topo I (McConaughy et al., 1981) and topo II (Osheroff et al., 1983). In contrast, substitution of Cl^- by Glu^- or Asp^- extended the range of processivity to approximately 100 mM (NaGlu or NaAsp) or 200 mM (KGlu or KAsp). It must be pointed out that NH_4Glu, with or without $MgCl_2$, induced processive pathways up to 200 mM.

Differential Effects of Magnesium Chloride and Magnesium Hemiaspartate on the Kinetics and Behavior of DNA Relaxation. The kinetics of DNA relaxation were determined with reaction buffers containing varying Mg^{2+} concentrations: $MgCl_2$ or $MgAsp_2$ in the presence of KCl or $MgAsp_2$ alone (Figure 5). In the presence of 100 mM KCl, the range of $MgCl_2$ or $MgAsp_2$ in which relaxation occured was roughly

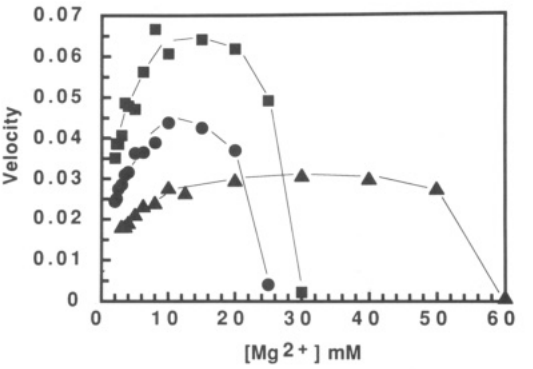


FIGURE 5: Initial velocity of *Candida* topo I relaxation as a function of $[Mg^{2+}]$. Incubation time = 2.5 min at 37 °C. ▲, $MgAsp_2$ alone; ■, $MgAsp_2$ + 100 mM KCl; ●, $MgCl_2$ + 100 mM KCl. Velocity = micrograms of relaxed DNA formed per minute.

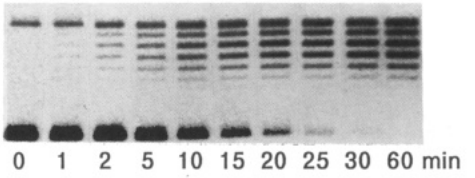


FIGURE 6: Magnesium effect throughout the entire time of topo I processive relaxation kinetics. Magnesium concentration = 25 mM ($MgAsp_2$). Incubation time = 0–60 min at 37 °C.

the same (up to 25–30 mM). The substitution of chloride ions by aspartate (as the magnesium counterpart) enhanced by 50% the optimal value for the velocity of the reaction. This could be explained by some unknown preference of topo I for aspartate compared to chloride when both are available. In these experiments, 100 mM KCl was responsible for a predominantly distributive reaction. In the absence of KCl, $MgAsp_2$ allowed DNA relaxation to occur and the Mg^{2+} range was extended to 60 mM. But in this range, the optimal value for inital velocity remained 3 times lower than with KCl. This may be explained by the mode of catalysis, which tends to be processive for all kinetics without the monovalent cation, thus slowing the turnover and the initial velocity (Figure 6). Substitution of $MgAsp_2$ by $MgGlu_2$ gave the same results, whereas $MgCl_2$ was poorly efficient alone (data not shown).

Plotting the velocity (V) against the monovalent ion concentration gave sigmoidal curves (Figure 2). In contrast, the velocity increased as a function of the magnesium concentration in the three plots with a simple hyperbolic progression until the plateau was reached (Figure 5). It was then possible to derive from the segment between 0 and 10 mM $[Mg^{2+}]$ of each plot a Lineweaver–Burk representation $[1/V = f(1/[Mg^{2+}])]$. The intercept with the abscissa $(1/[Mg^{2+}])$ was the same for the three experiments, which suggests magnesium binds to the enzyme or its substrate, DNA, independent of the other ions present in the reaction medium. This intercept gave an apparent K_d value of 2.5 mM; this is in large excess compared with the phosphate concentration in DNA (about 40 μM) and thus could not be evaluated at the molecular level (enzyme cofactor and/or DNA cofactor?).

Discussion

From the DNA relaxation kinetics shown here, several simple characteristics of the behavior of eukaryotic topo I can be identified. Initial velocity was always optimal when the reaction tended to be distributive, which means an efficient turnover. Glutamate and aspartate extended the range of the permissive salt concentration when they replaced chloride in both the processive and general reactions. They competed less than chloride with DNA to bind to the enzyme. Aspartate,

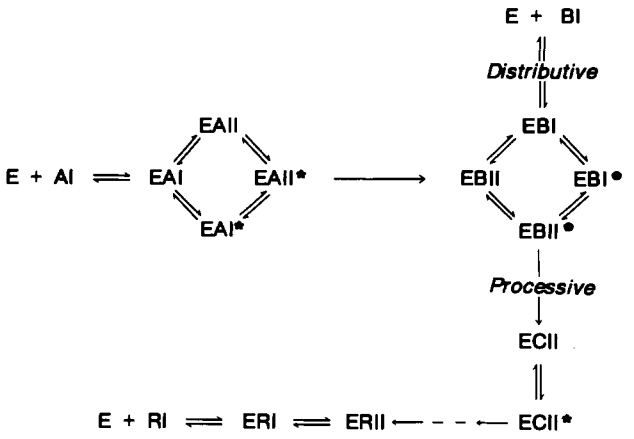
which was equally or even more efficient than glutamate, activated the initial velocity when it was diluted in a chloride solution. K^+ seemed to have a slightly greater effect on the overall rate and was more favorable for processivity than Na^+ and much more rate efficient but less favorable for processivity than NH_4^+. Except in the presence of NH_4Glu, magnesium was required to promote processivity; it could even engage this mode without monovalent cations when the anionic counterpart was aspartate or glutamate (not shown) but not chloride. Finally, magnesium could bind specifically to the enzyme–DNA complex, with an affinity that was independent of the composition of monovalent ions.

Our results give another example in which substitution of Cl^- by Glu^- or Asp^- promotes in vitro the stability of an enzyme–DNA complex, thus favoring the related catalysis. This phenomenon is probably related to the anion effects proposed by Record et al. (1985): anion displacement by DNA inside the DNA-binding site or anionic exclusion relative to water from the protein surface during topo I–DNA complex formation (Arakawa & Timasheff, 1984). Glu^- and Asp^- are considered to rank at the left end of the Hoffmeister anion series, when chloride is in the middle of the ranking. Even chloride is generally estimated as being relatively inert; its substitution by Glu^- or Asp^- may generate a better approximation of what must be in vivo the complete interaction between a given protein and its DNA site.

A complete processive reaction requires that DNA topo I associates with at least one of the numerous appropriate DNA sites, cuts one strand, induces the intact strand to pass through the break the number of times necessary to obtain complete relaxation, then religates the cut strand and finally dissociates from its binding site. Such a process must be carried out when complete catalysis is much faster than dissociation and is done experimentally by lowering the monovalent salt concentration and/or strength, which disfavors separation, and by adding magnesium. This gives the striking result that topo I may then become intimately associated with its binding site, which strongly slows down enzyme turnover, hereby explaining why conditions favoring the distributive mode are necessary to optimize the overall kinetics of DNA relaxation: free monovalent anions and cations are required in vitro to accelerate the dissociation of the topo I–DNA complex (McConaughy et al., 1981; Shuman & Prescott, 1990). This step and the resulting turnover of topo I are likely accountable for the first-order kinetics, and their variations are surely what we have measured under conditions of initial velocity (Pulleyblank & Ellison, 1982). In this context, NH_4Glu, which by itself surprisingly promotes processivity, was the poorest monovalent salt for rate stimulation: it seems to be the worst salt for turnover. Otherwise, in all the other salt combinations used here, magnesium was always required for processivity but was unequally stimulatory for the initial rate. Therefore, it seems reasonable to discriminate between it and the monovalent cations. While the monovalent cations influenced the equilibrium between association and dissociation, magnesium seemed essentially to accelerate the catalysis per se. It seems that this activity of magnesium competed with the monovalent cations present in the reaction medium, their progressive increase tended to switch to the distributive mode before attaining complete inhibition, when the increase of magnesium alone with aspartate as the anionic counterpart maintained processivity over the entire range of detectable activity.

According to our results and those recently reported on the topological impact on this catalysis (Camilloni et al., 1988, 1989; Caserta et al., 1990), we propose (Scheme I) the existence of an intermediate complex in the enzymic scheme of DNA relaxation catalyzed by a eukaryotic topo I (Pulleyblank & Ellison, 1982). It would be short-lived, topology-dependent, and magnesium-activated: a complex in which the enzyme traps the equivalent of a free supercoil. This complex would succeed the first noncovalent complex formed between topo I and its DNA site as an alternative complex to the well-known covalent one. Enzyme formation of the complex would be governed by the specific linking difference (the level of supercoiling) of the circular DNA involved and by the dynamic progression of the supercoils toward the DNA site. The complex would be formed whether the supercoil is negative or positive, which presumably requires a high conformational flexibility of the enzyme. This hypothesis should be viewed in light of the recent finding that, unexpectedly, a eukaryotic topo I preferentially interacts with intermolecular DNA crossovers (Zechiedrich & Osheroff, 1990). Relaxation of the trapped supercoil would occur if the enzyme couples trapping and nicking. The free energy emitted by this relaxation would irreversibly drive intact strand through the broken strand. The overall relaxation will be processive if the enzyme rapidly couples trapping another resting free supercoil with relaxing the previously captured one and does so the number of times necessary until the DNA becomes relaxed. This processive mode imposes on both the enzyme and the DNA some fast conformational changes: these would be supported by magnesium. This divalent ion may act at one of the two following levels (or both): binding to the enzyme with some anionic residues in the vicinity of the active site to promote trapping of a supercoil or, alternatively, changing the structure and dynamics of supercoiled DNA (Adrian et al., 1990). Whichever level is involved, religation of the transient break

Scheme I: Model of DNA Relaxation Catalyzed by a Eukaryotic Topoisomerase I and Its Two Alternative Pathways[a]



[a] A, B, and C are three supercoiled topoisomers. C has one supercoil less than B and two less than A. R is the relaxed form of the same DNA. (I, closed circular form; II, nicked intermediate covalently bound to E; *, enzyme-trapped supercoil.) After the initial binding (EAI), the enzyme may alternatively form a transient break coupled to a phosphotyrosine (EAII) or trap a supercoil (EAI*). Combination of both provokes a fast energy-driven step of relaxation that forms EBII. The reaction enters its distributive mode if closing (EBII → EBI) and dissociation (EBI → E + BI) are faster than trapping another supercoil (EBII → EBII*). On the contrary, if EBII* is produced quickly, then another relaxation step will occur (EBII* → ECII). The reaction is theoretically fully processive, if the DNA goes to its completely relaxed state without enzyme dissociation and, perhaps, without nick closure. We propose the transition EBII → EBII* as the key step differentiating between the two pathways: this transition must be strongly activated by the presence of magnesium, the stability of the complex, the extent of supercoiling, and the flexibility of the DNA-binding site. The level and the combination of these parameters will govern the catalytic mode undertaken by a eukaryotic topo I.

by the enzyme would be much easier only when the latter "stalls", i.e., when it stops trapping supercoils. Magnesium would then activate this religation, as has been shown previously by Shuman and Prescott (1990) using linear DNA.

In the case of the ATP-dependent DNA topoisomerases, the bacterial gyrase and the ultrathermophilic archaebacterial reverse gyrase, which introduce, respectively, negative and positive supercoils into the DNA, such a complex—in which a supercoil (or a part of it) is trapped by the enzyme—was easily isolated in vitro (Liu & Wang, 1978; Jaxel et al., 1989). But in these cases, ATP drives an energetically unfavorable reaction. It is then possible, by omitting ATP, to uncouple the initial binding of the enzyme and its resulting catalysis. The "topo-trapped DNA" complex is then a long-lived complex. On the other hand, for a eukaryotic topo I, uncoupling of this kind has never been observed. Catalysis is effective without ATP, even without magnesium, the driving force being in the supercoils that are relaxed. As a consequence, when a supercoil is trapped by such an enzyme, it can do nothing else but relax; the resulting complex must be short-lived. Thus, two ways are available to catch this complex: to markedly slow down catalysis while maintaining processivity (perhaps by using the specific inhibitor camptothecin or DNA minicircles) or to use fast methods, like UV laser cross-linking.

We have based the processive model on the simple and very classic assumption that a eukaryotic topo I requires, to be an efficient catalyst, an active capacity to propagate the topological constraint of its DNA substrate in the vicinity of the transient break (Maxwell & Gellert, 1986). To achieve this, the topo I binding site on the DNA and its flanking regions must have some appropriate flexibility: this may be an important feature that governs a DNA-binding site and is in good accord with the recent results obtained by Caserta et al. (1990) showing a reduction in the number of active sites during the course of DNA relaxation. Moreover, removing negative supercoils (increasing Lk) and positive supercoils (decreasing Lk) may involve two different flexible classes of DNA sites, because of the anisotropic properties of the double helix.

Although the reaction scheme presented is speculative, it is consistent with the data, draws a reasonable conclusion to our results, and is a prelude to future experiments. By continuing to study how to switch from distributivity to processivity, we may approach more accurately the basic function of eukaryotic topo I in vitro. It should be recalled that this enzyme is supposed to sit on appropriate DNA sites and to relax processively the waves of DNA supercoiling induced, at any time, in its nuclear domain by all the possible DNA-tracking processes (Liu & Wang, 1987; Caserta et al., 1990). Since this has already been shown in the context of the paradigmatic processive catalysis, DNA polymerization, components others than magnesium and weak monovalent anions should be able to help efficiently in vitro the processive relaxation: for example, other divalent cations, some specific proteins, or a polymer that provokes macromolecular crowding (PEG) (Forterre et al., 1985; Tan et al., 1986; Tabor et al., 1987; Jarvis et al., 1990). The different and complex post-translational modifications of the enzyme (Osheroff, 1989) as well as the relaxing flow (i.e., removing positive or negative supercoils) may also greatly influence this fascinating catalysis.

Acknowledgments

We are indebted to Professor Michel Duguet for the use of laboratory facilities. We thank Janet Jacobson for carefully reading the manuscript, Setha Douc-Rasy for valuable discussions, Claudine Templé for technical assistance, and Dominique Morineau for photographs.

Registry No. Asp, 56-84-8; Glu, 56-86-0; KGlu, 19473-49-5; NaGlu, 142-47-2; KAsp, 1115-63-5; NaAsp, 3792-50-5; $MgAsp_2$, 2068-80-6; $MgGlu_2$, 110011-84-2; NH_4Glu, 7558-63-6; KCl, 7447-40-7; Mg, 7439-95-4; NaCl, 7647-14-5; NH_4Cl, 12125-02-9; topoisomerase, 80449-01-0.

References

Adrian, M., ten Heggeler-Bordier, B., Walhi, W., Stasiak, A. Z., Staziak, A., & Du Bochet, J. (1990) *EMBO J. 9*, 4551–4554.

Arakawa, T., & Timasheff, S. N. (1984) *J. Biol. Chem. 259*, 4979–4985.

Badaracco, G., Plevani, P., Ruyechan, W. T., & Chang, L. M. S. (1983) *J. Biol. Chem. 258*, 2022–2026.

Been, M. D., & Champoux, J. J. (1984) *J. Mol. Biol. 180*, 515–531.

Bonven, B. J., Gocke, E., & Westergaard, O. (1985) *Cell 41*, 541–551.

Brill, S. J., & Sternglanz, R. (1988) *Cell 59*, 403–411.

Brun, G., Vannier, P., Scovassi, I., & Callen, J.-C. (1981) *Eur. J. Biochem. 118*, 407–415.

Busk, H., Thomsen, B., Bonven, B. J., Kjeldsen, E., Nielsen, O. F., & Westergaard, O. (1987) *Nature (London) 327*, 638–640.

Camilloni, G., Di Martino, E., Caserta, M., & Di Mauro, E. (1988) *Nucleic Acids Res. 16*, 7071–7085.

Camilloni, G., Di Martino, E., Di Mauro, E., & Caserta, M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3080–3084.

Caserta, M., Amadei, A., Camilloni, G., & Di Mauro, E. (1990) *Biochemistry 29*, 8152–8157.

Champoux, J. J. (1978) *J. Mol. Biol. 118*, 441–446.

Champoux, J. J., & Dulbecco, R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 143–146.

Clewel, D. B., & Helinsky, D. R. (1970) *Biochemistry 9*, 4428–4440.

Der Garabedian, P. A., & Vermeersch, J. J. (1987) *Eur. J. Biochem. 167*, 141–147.

Dynan, W. S., Jendrisak, J. J., Hager, D. A., & Burgess, R. R. (1981) *J. Biol. Chem. 256*, 5860–5865.

Eng, W., Pandit, S. D., & Sternglanz, R. (1989) *J. Biol. Chem. 264*, 13373–13376.

Forterre, P., Mirambeau, G., Jaxel, C., Nadal, M., & Duguet, M. (1985) *EMBO J. 4*, 2123–2128.

Giaever, G. N., & Wang, J. C. (1988) *Cell 55*, 849–856.

Goto, T., & Wang, J. C. (1984) *Cell 36*, 1073–1080.

Griep, M. A., & McHenry, C. S. (1989) *J. Biol. Chem. 264*, 11294–11301.

Jarvis, T. C., Ring, D. M., Daube, S. S., & von Hippel, P. H. (1990) *J. Biol. Chem. 265*, 15160–15167.

Jaxel, C., Nadal, M., Mirambeau, G., Forterre, P., Takahashi, M., & Duguet, M. (1989) *EMBO J. 8*, 3135–3139.

Keller, W. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4876–4880.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lazarus, G. M., Henrich, J. P., Kelly, W. G., Schmitz, S. A., & Castora, F. J. (1987) *Biochemistry 26*, 6195–6303.

Leirmo, S., Harrison, C., Cayley, D. S., Burgess, R. R., & Record, M. T., Jr. (1987) *Biochemistry 26*, 2095–2101.

Liu, L. F., & Miller, K. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3487–3491.

Liu, L. F., & Wang, J. C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2098–2102.

Liu, L. F., & Wang, J. C. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7024–7027.

Lohman, T. M., Chao, K., Green, J. M., Sage, S., & Runyon, G. T. (1989) *J. Biol. Chem. 264*, 10139–10147.

Lynn, R. M., Bjornsti, M., Caron, P. R., & Wang, J. C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3559-3563.
Maxwell, A., & Gellert, M. (1986) *Adv. Protein Chem. 38*, 69-109.
McConaughy, B. L., Young, L. S., & Champoux, J. J. (1981) *Biochim. Biophys. Acta 655*, 1-8.
Muller, M. T. (1985) *Biochim. Biophys. Acta 824*, 263-267.
Muller, M. T., Pfund, W. P., Mehta, V. B., & Trask, D. K. (1985) *EMBO J. 4*, 1237-1243.
Oakley, B. R., Kirsch, D. R., & Morris, N. R. (1980) *Anal. Biochem. 105*, 361-363.
Oddou, P., Schmidt, U., Knippers, R., & Richter, A. (1988) *Eur. J. Biochem. 177*, 523-529.
Osheroff, N. (1989) *Pharmacol. Ther. 41*, 223-241.
Osheroff, N., Shelton, E. R., & Brutlag, D. L. (1983) *J. Biol. Chem. 258*, 9536-9543.
Overman, L. B., Bujalowski, W., & Lohman, T. M. (1988) *Biochemistry 34*, 440-447.
Pulleyblank, D. E., & Ellison, M. J. (1982) *Biochemistry 21*, 1155-1161.
Record, M. T., Jr., Anderson, C. F., Mills, P., Mossing, M., & Roe, J. H. (1985) *Adv. Biophys. 20*, 109-135.
Richey, B., Cayley, D. S., Mossing, M. C., Kolka, C., Anderson, C. F., Farrar, T. C., & Record, M. T., Jr. (1987) *J. Biol. Chem. 262*, 7157-7160.
Schaffner, W., & Weissman, C. (1973) *Anal. Biochem. 56*, 502-514.
Shaffer, R., & Traktman, P. (1987) *J. Biol. Chem. 262*, 9309-9315.
Shuman, S., & Prescott, J. (1990) *J. Biol. Chem. 265*, 17826-17836.
Shuman, S., Golder, M., & Moss, B., (1988) *J. Biol. Chem. 263*, 16401-16407.
Tabor, S., Huber, H. E., & Richardson, C. C. (1987) *J. Biol. Chem. 262*, 16212-16223.
Tan, C. K., Castillo, C., So, A. G., & Downey, K. M. (1986) *J. Biol. Chem. 261*, 12310-12316.
Thrash, C., Bankier, A. T., Barrell, B. G., & Sternglanz, R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4374-4378.
Tricoli, J. V., & Kowalski, D. (1983) *Biochemistry 22*, 2025-2031.
Uemura, T., Morino, K., Shiozaki, K., & Yanagida, M. (1987) *Nucleic Acids Res. 15*, 9727-9739.
Vosberg, H. P., Grossman, L. I., & Vinograd, J. (1975) *Eur. J. Biochem. 55*, 79-93.
Wang, J. C. (1985) *Annu. Rev. Biochem. 54*, 665-697.
Wu, H. Y., Shyy, S., Wang, J. C., & Liu, L. F. (1988) *Cell 53*, 433-440.
Zechiedrich, L. F., & Osheroff, N. (1990) *EMBO J. 9*, 4555-4562.
Zwelling, L. A., Chan, D., Hinds, M., Silberman, L., & Mayes, J. (1988) *Biochem. Biophys. Res. Commun. 152*, 808-817.

Receptor-Modulated Iron Release from Transferrin: Differential Effects on N- and C-Terminal Sites†

Pawan K. Bali‡ and Philip Aisen*,‡,§
Departments of Physiology and Biophysics and of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461
Received May 15, 1991; Revised Manuscript Received July 31, 1991

ABSTRACT: Iron release to PP_i from N- and C-terminal monoferric transferrins and their complexes with transferrin receptor has been studied at pH 7.4 and 5.6 in 0.05 M HEPES or MES/0.1 M NaCl/0.01 M CHAPS at 25 °C. The two sites exhibit kinetic heterogeneity in releasing iron. The N-terminal form is slightly less labile than its C-terminal counterpart at pH 7.4, but much more facile in releasing iron at pH 5.6. At pH 7.4, iron removal by 0.05 M pyrophosphate from each form of monoferric transferrin complexed to the receptor is considerably slower than from the corresponding free monoferric transferrin. However, at pH 5.6, complexation of transferrin to its receptor affects the two forms differently. The rate of iron release to 0.005 M pyrophosphate by the N-terminal species is substantially the same whether transferrin is free or bound to the receptor. In contrast, the C-terminal form releases iron much faster when complexed to the receptor than when free. Urea/PAGE analysis of iron removal from free and receptor-complexed diferric transferrin at pH 5.6 reveals that its C-terminal site is also more labile in the complex, but its N-terminal site is more labile in free diferric transferrin. Thus, the newly discovered role of transferrin receptor in modulating iron release from transferrin predominantly involves the C-terminal site. This observation helps explain the prevalence of circulating N-terminal monoferric transferrin in the human circulation.

The predominant pathway for uptake of iron by most vertebrate cells, receptor-mediated endocytosis of transferrin, involves two glycoproteins: transferrin and the transferrin receptor (Klausner et al., 1983; Dautry-Varsat et al., 1983). Transferrin, which functions to transport iron in the circulation, consists of a single 80-kDa polypeptide chain arranged in a bilobal structure. Each lobe is comprised further of two domains surrounding a cleft bearing a specific high-affinity iron-binding site (Anderson et al., 1987; Bailey et al., 1988). Although similar, the two sites differ in accessibility to iron chelates, binding strength, and spectroscopic properties (Aisen et al., 1978); kinetic lability (Baldwin & de Sousa, 1981; Bali

† This work was supported in part by Grant DK15056 from the National Institutes of Health.
* To whom correspondence should be addressed.
‡ Department of Physiology and Biophysics.
§ Department of Medicine.

0006-2960/91/0430-9947$02.50/0