Renaturation of Denatured, Covalently Closed Circular DNA*

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William Strider,† Merrill N. Camien, and Robert C. Warner

From the Department of Molecular Biology and Biochemistry, University of California, Irvine, California 92717

The rate of renaturation of denatured, covalently closed, circular DNA (form I, DNA) of the φX174 replicative form has been investigated as a function of pH, temperature, and ionic strength. The rate at a constant temperature is a sharply peaked function of pH in the range of pH 9 to 12. The position on the pH scale of the maximum rate decreases as the temperature is increased and as the ionic strength is increased. The kinetic course of renaturation is pseudo-first order: it is independent of DNA concentration, but falls off in rate from a first order relationship as the reaction proceeds. The rate of renaturation depends critically on the temperature at which the denaturation is carried out. Form I, prepared at an alkaline pH of 9°C, renatures from 5 to more than 100 times more rapidly than that similarly prepared at 50°C. Both the heterogeneity in rate and the effect of the temperature of denaturation depend, in part, on the degree of supercoiling of the form I DNA from which the form Iα is prepared. However, it is concluded that a much larger contribution to both arises from a configurational heterogeneity introduced in the denaturation reaction.

The renaturation rate was determined by neutralization of the alkaline reaction and analytical ultracentrifugal analysis of the amounts of forms I and Iα. The nature of the proximate renatured species at the temperature and alkaline pH of renaturation was investigated by spectrophotometric titration and analytical ultracentrifugation. It is concluded that the proximate species are the same as the intermediate species defined by an alkaline sedimentation titration of the kind first done by Vinograd et al. (1965) Proc. Natl. Acad. Sci. U. S. A. 53, 1104-1111.

Observations are included on the buoyant density of forms I and form Iα and on depurination of DNA at alkaline pH values and high temperatures.

The first observations on the formation of a unique, denatured species of covalently closed, circular DNA after exposure to an alkaline pH were made by Dulbecco and Vogt (1) on polyoma DNA. They characterized the species by its high sedimentation coefficient in alkali, its retention of a high coefficient when neutralized, and its renaturation by annealing at pH 11.8 in 6 M CsCl to yield a product with the sedimentation coefficient of the original polyoma DNA. The rapidly sedimenting species was further studied by Weil and Vinograd (2) who termed it a "cyclic coil." A similar species was identified by Pouwels and Jansz (3) when φX174-RF1 was denatured in alkali. However, it was not until the work of Vinograd et al. (4) that the relation of the cyclic coil to other allomorphic forms of circular DNA was clearly established. We will use the designation, form Iα, or simply Iα, for this species as a modification of the terminology introduced by Vinograd et al. (4). More specifically, form Iα will be used to refer to the species that sediments rapidly in alkali and retains, after neutralization, a sedimentation coefficient approximately twice that of form I. The distinction is necessary because there is a gradual change in buoyant density (5) and in sedimentation coefficient (4) as the pH is raised until the maximum value characteristic of form Iα is attained near pH 12.7. It was found by Pouwels et al. (10) that below this pH, neutralization resulted in the reformation of form I, whereas, above it, form Iα was stable. They studied a number of properties of Iα that, together with previous work (2, 4, 5), indicated that it has essentially all of its base pairing disrupted and has a compact, collapsed structure which derives from the plectonemic linkage of the two strands. The distinction between form Iα and the partially denatured molecules, with respect to the change in sedimentation coefficient during a titration of form I DNA, was more precisely defined by Rush and Warner as a basis for their preparative method for circular DNA (11). The specific point in the titration at which form I can no longer be recovered by neutralization defines the point at which the last complementary stretch of DNA capable of nucleating a renaturation is titrated. Similar observations have been made by Ostrander and Gray on PM2-DNA (12).

The Iα formed at high pH values requires exposure to an annealing environment in order to initiate nucleation and consequent renaturation. This was clearly demonstrated by the experiment of Dulbecco and Vogt (1) referred to above. An examination of some conditions for renaturation was made by Pouwels et al. (13). They found that heating at pH 12.6 at 50°C for 1 h at low ionic strength, or at lower temperatures at lower pH values and higher ionic strengths for shorter times resulted in renaturation yielding form I DNA. In a recent study, Lau and Gray (14) have investigated the renaturation of several I1 DNAs mediated by EtdBr in strongly denatured form I DNA.

The abbreviations used are: RF, replicative form; form I, covalently closed circular, duplex DNA; form Iα, denatured form I DNA; form Iβ, relaxed form I DNA; form II, superhelical form I; form IIα, nicked form I; EtdBr, ethidium bromide.

*This species was previously referred to as "form IV" (6-8). It has been suggested (J. C. Wang, personal communication) that "form I" should be used to designate covalently closed, circular duplexes of any configuration and that, where necessary, a subscript be added to indicate particular variations on the parent structure. In this paper, we use Iα for the denatured, I for the relaxed species, and I to indicate superhelicity where necessary. In the absence of a subscript, "form I" should retain its present common usage of referring to a species that is supercoiled to the degree found in naturally occurring circular DNA. This change will prevent confusion with the recent use of "form IV" for relaxed DNA; for example, in Ref. 9. Such usage should also be discontinued.

†Present address, New York University School of Medicine, New York, NY 10016.
alkaline solution.

Our initial interest was in taking advantage of the high sedimentation coefficient of $\phi X 174$ DNA in preparative procedures for which optimum, reproducible conditions for renaturation were required. A systematic study of the effect of pH and temperature on renaturation was undertaken and a preliminary report of the results was published (6). The study was expanded, but a comprehensive account has been delayed for investigation of the surprising result that the kinetics of the reaction is not first order in spite of its evident intramolecular nature. Some aspects of the renaturation of various circular DNAs and applications of renaturation to preparative procedures have meanwhile been reported (7, 8, 15). We describe here the results of a study of the effects of pH, temperature, salt concentration, superhelicity, and the influence of the conditions of denaturation on the rate of renaturation, as well as other studies bearing on the mechanism of the reaction. The work reported at this time is limited to studies of monomeric $\phi X 174$-RF.

EXPERIMENTAL PROCEDURES

RESULTS

Effect of pH and Temperature—The dependence of the extent of renaturation after heating $\phi X 174$ DNA at various pH values for a fixed time is shown in Fig. 1. The results display the combinations of pH and temperature that will establish annealing conditions under which a nucleation of the renaturation reaction can be initiated. At each temperature there is an optimum pH and a sharp decrease in rate on either side. In previous examinations of the renaturation of form $\phi X 174$ (1, 13), the conditions investigated were too limited to reveal this interdependence of pH and temperature in determining the rate of the reaction. Results at other temperature and pH values are given in the miniprint supplement.

The effect of salt concentration on the rate is shown in Fig. 2. The pH optimum is moved to a higher pH by a reduction in ionic strength. This shift is in the same direction as the change in $\nabla m$, the point of half-titration, with ionic strength of the alkaline branch of a DNA (16) or a poly(rA)-poly(rU) (17) titration curve.

The time course of the reaction was determined under several conditions chosen to have rates low enough to be determined by the methods we have used to start and stop the reaction. A typical example is shown in Fig. 3 in which the data are presented as a logarithmic plot. The rate of the reaction falls off with time from that required for first order kinetics. In spite of this, the rate is seen to be independent of the initial concentration of DNA, and thus, to be intramolecular as would be expected. A heterogeneity in rate of this kind may have its origin in molecular heterogeneity or in a complexity of the reaction mechanism. Evidence is presented below that the process of denaturation itself gives rise to a population of configurations differing, under renaturing conditions, in the probability that a nucleating event leading to renaturation will take place. This molecular heterogeneity is
one that characterizes the denatured state, but is not inherent in form I DNA. The possibility that heterogeneity of the latter kind contributes to the character of the renaturation kinetics is also examined; in particular, that the established heterogeneity of form I with respect to superhelical density (18, 19) has an effect. One possible type of complexity in reaction mechanism, that of accumulation of an intermediate, is also considered.

Effect of the Conditions of Denaturation—The configurational heterogeneity of form I, DNA was inferred in part, from experiments in which the effect on the rate of renaturation of varying the temperature of alkaline denaturation of form I was examined. Of a number of conditions of denaturation tried, only temperature had a significant effect. Those conditions not resulting in a change in rate were: 1) pH as long as it was above the value at which all of the form I was converted to form II; 2) time of exposure to the alkaline pH beyond that used in our standard procedure; and 3) salt concentration at the alkaline pH.

The striking effect of temperature is shown in Fig. 4. The rate for the 0°C-denatured DNA (0°C-Id) was too fast to obtain a satisfactory rate constant under these conditions, but is seen to be 20 to 100 times the rate for 50°C-denatured DNA (50°C-Id) over the range of renaturation of the latter plotted in Fig. 4. Intermediate rates were obtained at 0°C and 50°C yielded products having intermediate rates when the temperature was reduced below 25°C (Fig. 5). In the range between 25°C and 50°C, the Id DNA renatured at about the same rate. Experiments above 50°C were unsatisfactory because the formation of single and double strand breaks became significant. The large difference in the rate of renaturation of 0°C-Id relative to that of 50°C-Id is also evident in Figs. 3s and 4s. For example, 0°C-Id was the only form I that did not require an alkaline pH for renaturation. At pH 8 and 95°C, no renaturation of 25°C-Id or 50°C-Id could be observed, but 0°C-Id DNA was renatured with an initial rate constant of about 11 ks⁻¹ as shown in Fig. 5; Additional experiments, both at neutral and alkaline pH values, are described in the miniprint supplement. These results demonstrate that much greater differences in the renaturation rate can be introduced by manipulation of the temperature of denaturation than are characteristic of the variation of rate with time in a single kinetic experiment such as that shown in Fig. 3.

Effect of Supercoiling—Differences in superhelical density provide the only known source of molecular heterogeneity of form I DNA (19). Superhelical density affects the buoyant density (5, 20) and sedimentation coefficient (21) of form II, and the buoyant density of intermediates in the alkaline denaturation of I to II (20). A decrease in superhelical density (lower Δφ) has been shown to decrease the rate of Endo III-mediated renaturation of I, DNA in alkali (14). Superhelical density might thus affect the configuration of I in a way that would be reflected in its rate of renaturation and would contribute to the heterogeneity in the rate constant for renaturation. In order to evaluate this effect, it first was shown that the heterogeneity of superhelical density of form I of φX-RF is comparable to that of other form I DNAs (22). Form I, was prepared from this DNA by a limit digest at 37°C with topoisomerase I. The product was shown to be relaxed and to differ from the I, from which it was prepared by a Δφ of about 30 turns (see miniprint supplement).

The I, DNA was used to prepare 0°C-Id and 50°C-Id. These were renatured in parallel with renaturation of 0°C-Id and 50°C-Id prepared from native supercoiled form I,. The two 50°C-Id DNAs were compared at 50°C and pH 10.95 (see "Experimental Procedures"). Curve a, DNA denatured at 50°C, , denatured at 25°C; curve b, denatured at 10°C; curve c, denatured at 0°C.

Fig. 5. Renaturation of I, DNA denatured at different temperatures and renatured under standard conditions at 50°C and pH 11.35 (see "Experimental Procedures"). Curve a, , denatured at 50°C, , denatured at 25°C; curve b, denatured at 10°C; curve c, denatured at 0°C.

Fig. 4. Comparison of the rates of renaturation of form I, DNAs that were denatured at 0°C and 50°C. Renaturation was carried out under standard conditions at 50°C and pH 10.95 (see "Experimental Procedures"). a, DNA denatured at 50°C, initial rate is about 45 ks⁻¹, dropping to about 7 ks⁻¹; b, DNA denatured at 0°C, the initial rate is about 850 ks⁻¹. The DNAs were denatured at the indicated temperatures as described under "Experimental Procedures" and were renatured at a concentration of about 60 μg ml⁻¹.
The difference in renaturation rates between the 50°-I₀ DNA samples prepared from I₀ and I₁ was smaller than those shown in Fig. 6 (curves not shown). As a result, a similar calculation to that above indicates that, for 50°-I₀, a smaller fraction of the heterogeneity can be attributed to supercoiling than for 0°-I₁. These estimates have only an order of magnitude precision and are intended to show that, although supercoiling has a significant effect, it cannot account for all of the rate heterogeneity in the kinetic course of renaturation.

The second effect that must be considered is the contribution of the difference in supercoiling resulting from the difference in temperature of denaturation of 0°-I₁ and 50°-I₀ prepared from the same I₀. A calculation from the temperature coefficient of supercoiling (23) indicates a difference of eight turns for a 50-degree difference in temperature. The increase in rate of 0°-I₁ due to a ΔT of ~30 turns at the time of denaturation is shown in Fig. 6. This was the maximum effect observed. It may be contrasted with the larger increase in rate between 50°-I₀ and 0°-I₀ for a ΔT of ~8 turns shown in Fig. 4, with the difference in initial rates shown in Fig. 5; or with the approximate differences in rates at a number of pH values shown in Figs. 3a and 4a. The same conclusion is reached as for the first effect. The contribution of supercoiling is substantial, but only accounts for a part of the effect of temperature on the rate of renaturation.

Evidence that heterogeneity is introduced into form I₀ in the denaturation process at constant temperature was obtained by a direct test not involving comparison of the effect of temperature differences during denaturation. A standard annealing mixture of 50°-I₀ was held at 40 °C to the point of half-renaturation. It was neutralized and separated into form I and I₀ fractions by velocity centrifugation at 20 °C on preformed CsCl gradients. The form I was purified by removal of contaminating I₀ on a nitrocellulose column (11). The form I₀ fraction was completely renatured using the optimum 70 °C conditions shown in Fig. 1. The resulting form I₀ and the form I₀ isolated from the CsCl gradient were then compared after denaturing them under identical conditions of time, temperature, and alkaline pH. No difference could be detected in the rates of renaturation of the two form I₀ DNAs obtained in this way. In addition, the I₀ DNA isolated from the 40 °C reaction mixture, when again subjected to renaturation under the initial 40 °C conditions, did so at a rate somewhat slower than found for the slow segment of an initial renaturation curve of the 50°-I₀ used in this experiment. This is in contrast to the same form I₀ which reproduced the entire range of rates when it was renatured at 70 °C, denatured, and its renaturation rate then measured. This experiment was also done using a 50 °C renaturation of 50°-I₀ with the same results.

We conclude that the heterogeneity in kinetics as illustrated in Fig. 3 is mainly due to heterogeneity of configuration of I₀ established in the denaturation reaction.

**Spectrophotometric Titration**—In the conversion of form I to I₀, the pH must be raised to a value well above the pH range in which renaturation can be observed (see miniprint section) in order to have complete denaturation. When the pH is adjusted to the range in which renaturation takes place at a given temperature (Fig. 1), conditions are established under which some proximate renatured species is the thermodynamically favored form. In our procedure for measuring the rate, the reaction mixture is neutralized to stop the reaction and any species that yields form I₀ in this procedure is considered to be renatured. At the pH and temperature of the renaturation reaction, it may be wholly or partially double-stranded, but at least it has undergone a rate-limiting reaction leading to renaturation. Experiments were undertaken to investigate the nature of the proximate species and its relation to forms I and I₀ and to the intermediate species between them defined by sedimentation titrations of the kind done by Vinograd et al. (4).

One of the ways in which the renaturation reaction can be examined at the pH and temperature at which it is occurring, i.e., without neutralization, is by spectrophotometric titration at a temperature and through a pH range in which renaturation can be observed. Such titrations of forms I and I₀ were performed at 25 °C, 40 °C, 50 °C, and 60 °C. The curves for 40 °C are displayed in Fig. 7. Renaturation is shown in the curve for I₀ by the time-dependent drop in absorbance when the pH was raised to 11.58. A similar sharp discontinuity could not be seen in the titrations at other temperatures. At 25 °C, the renaturation reaction was too slow and the pH at which it occurred so high that the difference between the A₅₀₀ values of forms I and I₀ was small. At 50 °C and 60 °C, the reaction was so fast that it occurred as rapidly as NaOH was added. No time-dependent drop in pH could be observed, but the I and I₀ curves coincided from the middle of the titration onward, indicating that renaturation was complete before the high pH end of the titration was reached. The decrease in A₅₀₀ at pH 11.58 shown in Fig. 7 occurred over a period of 30 min. The rate of change of A₅₀₀ became slow at this time and NaOH addition was resumed. The precise pH at which the decrease occurs under these titration conditions depends on the rate of addition of NaOH and on the size of the last addition of titrant before the drop in A₅₀₀. In order to show that it occurs in the region of the optimum pH defined by the curves in Figs. 1 and 2, it is necessary to correct for the change
in the pH of the buffer with temperature and the change in optimum pH with the ionic strength. These corrections are discussed in the miniprint section and the resulting estimate of the optimum pH at the ionic strength and temperature of the titration is indicated by the arrow at pH_{H_2O,25} = 11.65 in Fig. 7. We interpret the drop in absorbance accompanying renaturation to show that the molar absorbance of the proximate renatured species at the pH of renaturation is the same as that of form I brought to that pH. In order to make the same comparison with respect to hydrodynamic properties of the proximate renatured species with form I, the spectrophotometric curve is superimposed on a sedimentation titration in Fig. 7s. The superposition involves a number of corrections of the pH with respect to temperature and salt concentration because of the different conditions under which the two kinds of experiments were done (see miniprint supplement). Fig. 7s shows that the renaturation occurs in the pH range of the reversible portion of a sedimentation titration as defined by the titrations of Rush and Warner (11). This conclusion is supported more directly by sedimentation velocity experiments performed at high pH values at or near the temperature where renaturation occurs. These experiments are detailed in the miniprint supplement and sedimentation coefficients are given in Table IIs. These results define the proximate renatured species as one having the same molar absorption and the same sedimentation coefficient as form I at the pH of renaturation.

**DISCUSSION**

A summary of the pH optima for the rate of renaturation at several temperatures and ionic strengths is given in Table I, including a correction for the pH to its value at the temperature at which renaturation was carried out. For comparison, some data are shown for the alkaline pH values of nucleosides and of poly(U); these values are well below the pH optima. They shift with temperature and ionic strength in the same direction as the optima, but roughly half as much, so there is not a requirement for a constant degree of ionization for a maximum renaturation rate. The pH optima are also well above the pH at which linear or form II DNAs dissociate, as shown most directly by determinations of T_{m0} in alkaline buffers (26, 27), by sedimentation velocity titrations (28), or by buoyant density titrations (5).

In form I DNA, the topological restriction on unwinding results in a restriction on deprotonation evident in the titrations shown in Figs. 7 and 7s and measured by the alkaline buoyant density titrations of Vinograd et al. (5) and Wang (20). None of these titrations is completed until the pH is well above 12. The thymine and guanine bases in L DNA may be completely ionized at some renaturation pH values, but these pH values are all in a range in which, in covalently closed DNA, some protonated H-bonded regions of the partially renatured proximate species are stable. This comparison confirms the relation of the position of renaturation to the titration curves in Figs. 7 and 7s. The centrifugation experiments under renaturation conditions similarly show that the sedimentation coefficient of the species formed at the renaturation pH is the same as that of form I brought to that pH without having been denatured. A continuous series of intermediate, partially denatured species is demonstrated by each of the three types of alkaline titrations discussed above. The species have values for the property measured in a titration that are intermediate between those of forms I and L. The titrations are reversible up to the point at which L is formed, and the form I obtained by reversing a titration below this point is indistinguishable from native form I with respect to all properties that have been examined. We have used a preparative method (11) involving a reversible high pH treatment for \phi X174 and G4 RFs for a number of years and at various times have compared the properties of the monomer form I with that obtained by methods employing buoyant density separations, but no alkali treatment, with respect to sedimentation coefficient, extinction coefficient at 260 nm, buoyant density, specific infectivity in spheroplasts, and hydrolysis by several restriction enzymes. We have found no differences between these form I DNAs or between them and form I obtained by renaturation of L (data not shown). We conclude that the pH of renaturation of form L is within the pH range defined by the reversible region of an alkaline titration at the temperature of renaturation, and that the proximate renatured species has the same properties as the corresponding intermediate in the reversible phase of such a titration.

The pH at which L is formed in our standard procedure is 12.52. This pH at 25 °C is about 0.3 of a unit above the pH at which neutralization will yield form I and about 0.4 of a unit above the maximum pH at which renaturation can be observed at 25 °C. The properties of L at that pH are those defined by the alkaline titrations referred to above. When L is neutralized, its sedimentation coefficient drops to about \( s_{20,w} = 35 \) S (Fig. 7s), and its A_{260} relative to form I to about 1.1 (Fig. 7). These values are stable and reversible in subsequent exposure to alkali except under conditions in which renaturation takes place such as shown in Fig. 7.

The experiments at 80 °C and 95 °C (Fig. 4a) bring the renaturation pH into the neutral range and the temperature to a value at which there is denaturation of linear DNA without deprotonation. Form I cannot be denatured by heat to produce a stable denatured product such as L, in spite of the fact that the secondary structure is entirely disrupted as judged by the change in A_{260} (5). It thus behaves operationally as snapback DNA. However, there are not necessarily any residual helical regions present at the high temperature. Re-
nutation may occur rapidly on cooling, not because nucleated, helical regions are present, but because they form rapidly enough on cooling not to be rate-limiting. Renaturation of Iₜ under this condition might therefore be expected. It occurs only with the rapidly renaturable 0°-L₀ the barrier to renaturation of 50°-Iₚ appears to be kinetic rather than thermodynamic.

Lau and Gray (14) have reported extensive studies on EtdBr-mediated renaturation of form Iₜ of φX174, PM2, and Ab₂,b,DNA. A time-dependent reaction occurring under alkaline denaturation conditions was measured by neutralization and centrifugal analysis. The reaction appears to be the accumulation of a rapidly renaturable complex of DNA and EtdBr. On neutralization this complex presumably becomes nucleated so rapidly with respect to the formation of a base-paired product that an annealing step cannot be detected. They interpret the reaction to be a change in conformation in form Iₜ in which complementary bases are “apposed” without actually forming paired, nucleated regions. This sequence of renaturation steps is different from that discussed here in that the time-dependent reaction we have studied is an annealing step that takes place under conditions of pH and temperature at which a partially base-paired duplex is stable. The two types of renaturation are similar in that both require a conformational change in Iₜ driven by EtdBr binding in one case and by the energy of duplex formation at a certain pH and temperature in the other. More information will be needed on the binding of EtdBr to DNA in alkaline solution before a more direct comparison can be made.

Ostrander and Gray (12) found that a small fraction of form Iₜ of PM2-DNA renatured on neutralization of a strongly alkaline solution without exposure to annealing conditions. Lau and Gray (14) have shown that such Iₜ was fully denatured, was not cross-linked, and that the fast-renaturing DNA was not a unique fraction of PM2-DNA. It appears that some configurations can be locked in the Iₜ structure in such a way that, on neutralization, nucleation is no longer rate-limiting. The remaining fraction of the Iₜ DNA requires exposure to EtdBr (14) or to suitable pH and temperature conditions to renature. The structural basis for this phenomenon is not clear. We have described a similar behavior of chloroplast Iₜ-DNA (8) and have found other instances in which structural or sequence differences may modify the renaturability of Iₜ-DNA. One, apparently dependent on molecular weight, was reported briefly (7); another is a difference between 0°-Iₜ from φX174- and G₄-RFs that is presently under investigation.

The Denaturation and Renaturation Reactions—The stability of form Iₜ after neutralization has been attributed by Pouwels et al. (10, 13) and by Lau and Gray (14) to a shift or translocation of the complementary strands with respect to each other along the helix axis. We have used this concept in describing the renaturation reaction in terms of a rate of nucleation. A more detailed mechanism for the main features of renaturation and for the aspects of denaturation that give rise to the differences in the rate of renaturation and in the heterogeneity of renaturation rates can only tentatively be specified. An interpretation is offered here based in part on the discussion by Bauer and Vinograd (29) of the alkaline titration of form Iₜ and of the molecular nature of form Iₜ, modified by the results of the alkaline buoyant density titration of Wang (20) of forms Iₜ and Iₜ of PM2 DNA. The terminology of Bauer and Vinograd (29) for winding numbers and density of winding numbers as modified by Bauer (23) will be employed. In the coil regions of partially denatured form Iₜ, and in form Iₜ, the β windings have a different character than in helical regions where their pitch is determined by the strong interactions of the duplex structure. In coil regions, they are simply windings of one strand about the other. The two kinds of β windings will be characterized as βₐ and βₜ for helix and coil regions, respectively, each with its own local density of windings (11, 21, 29). On this basis, in the helical region of a partially denatured form Iₜ, the βₜ windings will have a density of about unity. The coil regions will not have a fixed value of βₜ. It can vary from zero up to a maximum that is determined by the number of times non-interacting strands can wind about each other without forcing the formation of positive supercoils. At this maximum, βₜ turns must have an average of more than 10 base pairs per turn and a density of less than unity if the coil regions are to acquire left-handed (+) supercoils as they are assumed to do in the discussion of Bauer and Vinograd (29), and in that below. It is recognized that the distribution between secondary, βₜ windings and tertiary or superhelical, τ, windings is in part dependent on the spatial writh of the molecule, but this distinction is not essential to the discussion.

In order to include the effect of supercoiling on aspects of Iₜ configuration that determine the rate of renaturation, the alkaline titration of a Iₜ and a Iₜ molecule will be considered. It is most convenient to begin the comparison when Iₜ has been titrated to the point of loss of supercoils where it has the sedimentation coefficient of form II. Here, the “early melting” (29) is approximately completed and the molecule contains a denatured loop without any windings. In contrast, Iₜ, as judged by the results of Wang (20), has lost no protons and presumably has retained the hydrodynamic properties it had at neutrality. As titration continues, Iₜ retains its initial buoyant density and does not lose protons until Iₜ is almost half-titrated (20). The two species must thus be compared with respect to the subsequent introduction of positive supercoils at similar degrees of deprotonation rather than at the same pH. As Iₜ, loses protons from the relaxed configuration, the newly formed coil regions have a low βₜ density because the number of βₜ turns is averaged over the just-dissociated helical region, which contributes a β density equal to about unity, and the existing coil, which has no windings. This lower average βₜ density (<1) will drive the formation of fewer positive supercoils than in Iₜ, in which, at a comparable deprotonation, the β density remains approximately at unity. There is a greater gain in entropy in Iₜ than in Iₜ for the reduction in βₜ by the introduction of a positive supercoil. This occurs at a lower pH for Iₜ, and the effect of supercoiling is reflected in the rise in sedimentation coefficient (4, 11) and in density (5, 20). Deprotonation of Iₜ occurs at a higher pH, has a much more cooperative character and results in the formation of the Iₜ structure over a very small pH span (20). No sedimentation velocity titration of a Iₜ DNA appears to have been done, but it would be expected to retain its initial sedimentation coefficient to a high pH. The greater superhelicity of Iₜ from Iₜ is presumably responsible for its greater sedimentation coefficient than that of Iₜ from Iₜ.

The definitions of the densities of windings in a molecule in which the numbers of windings are related by α = β + γ arise from the use of βₜ = N/10 as a normalizing factor for relating the winding numbers to a standard length of DNA, where N is the number of nucleotide pairs (23, 29). The density of βₜ windings will then be βₜ = 10βₜ / N. We wish here to consider a partially denatured molecule to consist of helix and coil regions of length Nₜₗ and Nₜc, respectively. If the helical region is essentially B-form DNA, it will be characterized by βₐ = Nₚₜ /10 and by a density of βₜ windings, Eₜ = 10βₜ / Nₜc = 1 (21, 29).

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The point to be addressed with respect to the relation of the \(L_0\) structures to their renaturability is the displacement or translocation of a locus on one strand from its complement on the other in the process of relieving \(\beta\) turns by the formation of positive supercoils, particularly in the final stages of this process. We are suggesting a correlation of rapid renaturability with lower positive superhelicity of form \(L_0\) and with a smaller displacement of strands. Thus, in 0°C-\(L_0\) and in \(L_0\) from \(L_1\), \(\tau\) would be less positive than in 50°C-\(L_0\) or \(L_0\) from \(L_1\), respectively. In each case, this would arise from a more negative \(\tau\) in the form \(L_1\) from which the respective \(L_0\) was prepared. Similarly, the distribution of negative supercoils in form \(L_0\) would give rise to a distribution of positive supercoils in \(L_0\) and thus contribute to the heterogeneity in the rate of renaturation. The major origin of the rate difference between 0°C-\(L_0\) and 50°C-\(L_0\) has been indicated to be a conformational difference arising from the difference in temperature at which the denaturations were carried out. In this process, near the alkaline end of the titration of form \(L_1\), positive supercoiling is extensive as judged from the results of sedimentation titrations (4, 11, 29). The last complementary regions are titrated when the molecule is in a strained configuration that is expected to differ among molecules because of thermal fluctuations during the accumulation of positive supercoils in the early phases of the denaturation. The titration of this last region then permits a displacement driven by the energy of the strained configuration and its partial relief by the redistribution of windings between \(\beta\) turns and +\(\tau\) turns. A number of factors that could determine the extent of this displacement may depend on the temperature. These include thermal fluctuations in configuration at the time of redistribution; torsional and rotational movements of the two single strands with respect to each other; and possible random variations fixed in different molecules during the early phases of positive supercoiling. These effects could give rise to a temperature-dependent degree of displacement and to a random fluctuation of the displacement at a constant temperature. Other interactions between the strands that could contribute to the stability of the \(\beta\) windings may be greater at 0°C than at 50°C. All such interactions would be subject to thermal fluctuations as a result of statistical variation in chain configurations and the randomization of a constant temperature to a population of species varying in degree of \(\beta\) winding, and resulting in the observed heterogeneity of the rate of renaturation.

In the renaturation process the probability of nucleation is assumed to be greater for a smaller initial displacement of complementary loci. Nucleation is facilitated when the variables of \(pH\) and temperature combine to produce a conformational change that is both partially protonated and has sufficient thermal flexibility that nucleated complementary regions will form and will lead to renaturation. A higher temperature is required to produce this situation when the DNA is more highly protonated. An optimum exists because, on the alkaline side, some correct nucleating events dissociate, and on the acid side, because incorrect nucleations are too stable.

With respect to existence of optimum conditions, this renaturation is similar to the much studied annealing of complementary, separate, single strands. Here, the rate-determining nucleating event is one established in a binucleotide encounter and, in contrast to the renaturation of \(L_0\), there is no dependence of \(pH\) over a neutral range and no restriction on the conformational flexibility of the single strands due to circularity and plectonemically linked character. However, an optimum temperature for formation of a successful nucleating event was shown by Marmur and Doty (30) in which changes in temperature play the same role as changes in \(pH\) in the renaturation of form \(L_0\) DNA.

Changes in Configuration of \(L_0\) after Denaturation—We have established that 0°C-\(L_0\) and 50°C-\(L_0\) have configurational differences that are reflected in their rates of renaturation. These properties are stable in neutral solution and reproducible as long as the standard conditions for denaturation and neutralization are adhered to. However, if neutral 0°C-\(L_0\) is raised to the \(pH\) for denaturation at 50°C, it becomes more slowly renaturable; that is, it assumes the character of 50°C-\(L_0\). We have not identified other conditions in which similar changes take place, nor is it clear whether they may proceed in the case of 0°C-\(L_0\) at renaturation \(pH\) values and temperatures. If so, they must be slower than renaturation or we would be unable to distinguish 0°C-\(L_0\) from 50°C-\(L_0\).

A few \(L_0\) preparations denatured under nonstandard conditions, for example, in alkaline sucrose gradients, have been examined that were slower to renature than 50°C-\(L_0\). We have not been able to reproduce such preparations satisfactorily, and it is evident that there are conditions not identified in the experiments that led to the establishment of our standard conditions, that can modify the renaturability of \(L_0\). None of these refractory preparations of \(L_0\) failed to renature rapidly under the optimum condition at 70°C shown in Fig. 1. These observations make it clear that the distribution of configurations present in \(L_0\) DNA are not so fixed at the time of renaturation that they cannot be modified subsequently by raising the temperature. The change in configuration, as expected, is in the direction of producing a species that renatures more slowly; that is, one with a more random structure and a greater translocation of strands. The same kind of change was observed by Lau and Gray (14) with respect to the fast-renaturing fraction of PM2-DNA discussed above. When PM2-\(L_0\) DNA prepared at 25°C was heated to 70°C, the fast-renaturing fraction disappeared. Similarly, \(\lambda\) \(b_2\) \(b_2\) DNA, which did not show a fast-renaturing fraction when denatured at 25°C, did so when denatured at 6°C. They also found differences in the EtdBr-mediated renaturation of \(\phi X174\), PM2, and \(\lambda\) \(b_2\) \(b_2\) \(L_0\) DNAs that depended on the temperature of denaturation. We have denatured PM2-DNA under our standard conditions at 0°C, 25°C, and 50°C and have found, in agreement with Lau and Gray (14), that the percentage of DNA renaturing on neutralizing the denaturation solution was temperature-dependent; the values were about 15%, 5%, and 0%, respectively, for the three temperatures. Lau and Gray (14) discuss some possible mechanisms that might favor configurational changes in \(L_0\) leading to more rapidly rather than less rapidly renaturable species. In our view, this could not take place in the absence of a perturbing ligand such as EtdBr or a change in conditions that would make base-pairing energetically favorable.

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SUPPLEMENT

Renaturation of Denatured, Covalently-Closed Circular DNA

William Strider, Merrill W. Cameron and Robert C. Warner

EXPERIMENTAL PROCEDURES

Phage RF-7H (serotype C) and phage RF174 are the same strains used in previous work except that RF was prepared by the same method with minor modifications (11). RF was used without fractionation in experiments since small amounts of form I and other species interfere only minimally with the centrifugal analysis. Nomenclature, form I, form II, and form III, is used in the literature to describe circular DNA isolated from Escherichia coli K-12. This was followed by a 51CrEDTA equilibrium gradient to remove form II and traces of host cell DNA. The velocity-centrifugation step was included to obtain purified DNA for other work and was not required for the experiments reported here.

Preparation Procedures - DNA was dialyzed against 2 M HCl, pH 2 and diluted with water to a concentration of about 200 ugi DNA per ml. Two volumes of this solution were mixed with one volume of a denaturation buffer of composition such that the final solution contained 0.1 M NaCl, 4.7 M LiCl, 1.5 M NaH2PO4, and 0.2 M Na2HPO4. All solutions were in the presence of 1000 ppm sodium azide. The resulting solution was chilled to 0°, and rapidly neutralized by adding an excess of 2 M HCl.NaCl.

The neutralized solution was dialyzed against 0.1 M HCl, pH 2 and concentrated by placing the dialysis tubing in dry ethanol at 5°C to yield a stock solution of about 100 ugi of form I DNA per ml in 0.1 M HCl. When it was found that the conditions of denaturation had a determining effect on the rate of renaturation, several variations on the above procedure were tried before it was adopted as the standard method. Neutralization of the DNA at the temperature of denaturation results in a larger sample that renatures less rapidly than when it was shifted to 0°C before neutralization. Mixing samples of DNA and denaturation buffer results in a DNA-DNA complex with different kinetic properties than the standard method. No effect of increasing the time of exposure to the denaturing temperature and pH could be detected.

Preparation Procedures - A buffer was prepared immediately before use from stock solutions of composition such that, when one volume was mixed with 2 volumes of LiCl, the resulting annealing solution contained 1 M NaCl, 2.07 M NaOH and 4.7 M LiCl. The phosphate was contributed by a mixture of 2.78M NaCl and 2.07 M LiCl in a ratio of 1:2 to yield the desired pH. A solution of this composition with a specified pH, and specified ionic strength, was added to aliquots of the denatured DNA to an extent sufficient to replace the NaCl lost in the procedure. No attempt was made to remove the unreacted phosphates because the rate of renaturation was not affected by this treatment. Aliquots of the denatured DNA were then mixed with the annealing solution to yield a final concentration of 100 ug DNA per ml in 0.1 M HCl. The samples were then mixed with 100 ug DNA per ml in 0.1 M HCl and 100 ugi of form I DNA per ml for centrifugal analysis.

As discussed in the text, rate curves are pseudo-first-order and cannot be characterized by a single rate constant. The continuous curves on the graphs of 20 T = T0 in terms of computer-calculation of the best fit for two components. The rate of reaction and the position of the components were treated as parameters, and they were not affected by any other parameters. The rate constant was a function of time from the lower to the upper limit, and the position of the components are given in units of natural logarithm and reciprocal logarithm (as).
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TABLE I

Data and factors for calculation of the change of pH of the renaturation solutions with temperature

| pH | 25°C | 30°C | 35°C | 40°C |
|----|------|------|------|------|
| pH | 1.0 | 1.5 | 2.0 | 2.5 |
| ΔpH | 0.3 | 0.5 | 0.8 | 1.0 |

The pH change was determined from the difference in pH at the indicated temperature from that at 25°C for solutions of the composition used for renaturation and titrations. All values may be applied to renaturation solutions in the range of ionic strengths 0.5 to 2.0 M.

Renaturation conditions: pH 7.0, 0.5 M NaCl, 0.1 M Na phosphate buffer, 0.01 M NaOH, 0.001 M NaCl. The pH initial range for all was from 6.0 to 9.0.

The results are given in Table I, which shows that the renaturation pH is increased by increasing the temperature from 25°C to 30°C. The pH change is significant and the increase is observed in all solutions between 25°C to 40°C, except for the solution containing 0.5 M NaCl, where the pH change is negligible.

The renaturation pH at 25°C and 30°C is given in Table I. The pH change is significant and the increase is observed in all solutions between 25°C to 40°C, except for the solution containing 0.5 M NaCl, where the pH change is negligible.

The effect of temperature on the renaturation pH is shown in Table I. The pH change is significant and the increase is observed in all solutions between 25°C to 40°C, except for the solution containing 0.5 M NaCl, where the pH change is negligible.

The effect of temperature on the renaturation pH is shown in Table I. The pH change is significant and the increase is observed in all solutions between 25°C to 40°C, except for the solution containing 0.5 M NaCl, where the pH change is negligible.

Fig. 2a: Gel electrophoresis of forms I and I'. The sample was released at 25°C in 0.1 M Tris-HCl buffer (pH 7.5) and the gel was stained with 0.2% Coomassie Blue R-250. The gel was then destained with 75% methanol and 10% acetic acid. The relative mobilities of forms I and I' were determined by comparing the migration distances of I and I' with a standard protein marker.

Fig. 2b: Gel electrophoresis of forms I and I', showing the renaturation of form I at 30°C. The sample was released at 25°C in 0.1 M Tris-HCl buffer (pH 7.5) and the gel was stained with 0.2% Coomassie Blue R-250. The gel was then destained with 75% methanol and 10% acetic acid. The relative mobilities of forms I and I' were determined by comparing the migration distances of I and I' with a standard protein marker.

Fig. 2c: Gel electrophoresis of forms I and I', showing the renaturation of form I at 30°C. The sample was released at 25°C in 0.1 M Tris-HCl buffer (pH 7.5) and the gel was stained with 0.2% Coomassie Blue R-250. The gel was then destained with 75% methanol and 10% acetic acid. The relative mobilities of forms I and I' were determined by comparing the migration distances of I and I' with a standard protein marker.

Fig. 2d: Gel electrophoresis of forms I and I', showing the renaturation of form I at 30°C. The sample was released at 25°C in 0.1 M Tris-HCl buffer (pH 7.5) and the gel was stained with 0.2% Coomassie Blue R-250. The gel was then destained with 75% methanol and 10% acetic acid. The relative mobilities of forms I and I' were determined by comparing the migration distances of I and I' with a standard protein marker.

In conclusion, the renaturation experiments at pH 7.0 attempts were made to see whether a stable form I' species could be made by heating at this pH. In confirmation of a number of observations in the literature, the renaturation pH was found to be around 7.0. In conclusion, the renaturation pH was found to be around 7.0. In conclusion, the renaturation pH was found to be around 7.0. In conclusion, the renaturation pH was found to be around 7.0.

Fig. 3a: Renaturation of 3'-deaminated I' at 80°C. The sample was released at 25°C in 0.1 M Tris-HCl buffer (pH 7.5) and the gel was stained with 0.2% Coomassie Blue R-250. The gel was then destained with 75% methanol and 10% acetic acid. The relative mobilities of forms I and I' were determined by comparing the migration distances of I and I' with a standard protein marker.
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