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Kinetic Analysis of the Annealing Period in the Formation of the Poly(A)·2Poly(U) Triple Helix

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Synopsis

The slow kinetics of annealing processes in multistranded nucleic acids is spectrophotometrically investigated using poly(A)·2poly(U) as a model system. The absorbance changes at specific wavelengths show that double-helical (A·U) base pairs appear as transient intermediates. The annealing process is identified by the enlargement of triple-helical sequences at the cost of (A·U) base pairs and unpaired (U) residues. A large time range in the reorganization of mismatched chain configurations is characterized by a logarithmic dependence on time. This observation is quantitatively described by a kinetic model developed by Jackson. In Jackson's model the rate-limiting process in the slow annealing phase of maximizing triple-helical sequences, is the removal of strand entanglements, knots, and hairpin loops by complete unwinding of those helical stretches which stabilize the mismatched configurations. The results of the present study are briefly discussed in terms of optimum conditions for hybridization experiments and for the preparation of polynucleotide complexes commonly used to produce interferons.

INTRODUCTION

Nucleic acid research frequently uses techniques which involve separation and recombination of complementary strands of multistranded polynucleotides. In this category, DNA–RNA hybridization experiments serve to measure the extent of relatedness between different single strands. Double-helical polynucleotide complexes used in studies on antiviral activity (see, e.g., Refs. 1 and 2) are usually prepared by mixing the single polymers under suitable experimental conditions of temperature, ionic strength, and pH. The initial phase of such complex formations is generally very rapid,[3–6] but the complete incorporation of all nucleotide residues in helical structures sometimes requires days of incubation. The slow recombination phase is commonly termed annealing period. It appears now that the production of maximum order and register in multistranded helices is an essential condition for many purposes. For instance, optimum antiviral activity may be achieved only after extended "tempering" of double-helical polynucleotide complexes.

In order to find out systematically the optimum conditions for the annealing of probably mismatched multistranded chain configurations, the mechanism of annealing processes in nucleic acids has to be investigated.

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A simple model reaction for such annealing processes is encountered in the slow phases of the formation of the three-stranded helix poly(A)·2poly(U) after mixing the single polymers polyriboadenylate, poly(A), and polyribouridylate, poly(U), at neutral pH and sufficiently high-ionic strength, in the molar ratio of the polymers 1:2.[7] The exact structure of base pairing in the (U·A·U) triple helix was recently given by Arnott and Bond.[8] The complex poly(A)·2poly(U) may consist of three individual strands or, alternatively, a part of one poly(U) strand may fold back onto an already existing double-helical stretch of (A·U) base pairs, thus forming triple-helical (U·A·U) sequences having only one poly(U) macromolecule interacting with poly(A).[9]

Although under favorable conditions of temperature, pH, and ionic strength, the formation of double- and triple-helical sequences is a rather rapid process, the complete incorporation of unpaired (U) and (A) residues into helices takes hours or days.[5]

The object of the present investigation is a kinetic analysis of a part of the slow formation of (U·A·U) sequences. We attempted to specify the commonly used term annealing by a specific structural reorganization. The extent of enlarging triple-helical stretches shows a characteristic time dependence, and the kinetic data can be quantitatively analyzed in terms of an unravelling model proposed by the late Julius Jackson. According to this quantitative approach, annealing of mismatched configurations to maximize the number of base pairs occurs by complete separation of helical stretches (unravelling).

MATERIALS AND METHODS

The polymers polyriboadenylate, poly(A), K-salt and polyribouridylate, poly(U), NH_4-salt (products of Miles Laboratories, U.S.A.) were separately dissolved in 0.05 *M* Na-Cacodylate-NaCl solution, pH 7.1 at 20°C. The various NaCl concentrations used were 0.1 *M*, 0.2 *M*, and 0.3 *M*, respectively. All solutions were filtered through 0.45-μm Millipore filter.

The concentration of the polynucleotides was determined spectrophotometrically after alkaline hydrolysis of the polymers.[10] The residue concentration of poly(A)·2poly(U) in the following experiments was $3 \times 10^{-5} M$(A)·2U).

Sterile conditions were maintained throughout all experimental phases in order to avoid contamination of the solutions by ribonucleases and bacteria.

The time course of absorbance changes after mixing the polymers was followed spectrophotometrically at specific wavelengths in a Zeiss-PMQ II spectrophotometer with thermostated cell holder. The sample cell was filled with 2.5-ml solution and the light path was 1 cm.

In the poly(A)–poly(U) system, absorbance changes at characteristic wavelengths are interpreted in terms of specific macromolecular processes such as helix–coil or helix–helix transitions. Here, the specific wavelengths are 260, 280, and 283.5 nm.[11,6]

In this framework the extent, ξ_U, to which (U) residues of poly(U) are incorporated in both helical structures, (A·U) sequences, and (U·A·U) stretches, can be derived from absorbance changes at 260 nm, A_{260}.

$$\xi_{(U)} = \frac{[(A \cdot U)] + 2[(U \cdot A \cdot U)]}{[U^0]} = \frac{A^0_{260} - A_{260}}{A^0_{260} - A^\infty_{260}} \tag{1}$$

In Eq. (1), $[U^0]$ is the total residue concentration of poly(U), A^0_{260} is the sum of the absorbances of the separated single polymers poly(A) and 2poly(U), and A^∞_{260} is the absorbance of completely base-paired polymers (no unpaired (U) residues).

The equilibration (U·A·U) $\rightleftharpoons$ (A) + 2(U) is selectively indicated by absorbance changes at 280 nm, A_{280}. The fraction, $\xi_{(U\cdot A\cdot U)}$, of (A) residues that is in triple-helical sequences is then given by

$$\xi_{(U\cdot A\cdot U)} = \frac{[(U \cdot A \cdot U)]}{[A^0]} = \frac{A^0_{280} - A_{280}}{A^0_{280} - A^\infty_{280}} \tag{2}$$

where $[A^0]$ is the total residue concentration of poly(A), A^0_{280} is the sum of the absorbances of the separated single polymers, and A^∞_{280} is the absorbance of the completely base-paired polymers.

In a similar manner the fraction of (A·U) sequences, $\xi_{(A\cdot U)}$, for the transition (U·A·U) $\rightleftharpoons$ (A·U) + U, can be calculated from absorbance changes at 283.5 nm, $A_{283.5}$.

$$\xi_{(A\cdot U)} = \frac{[(A\cdot U)]}{[A^0]} = \frac{A^0_{283.5} - A_{283.5}}{A^0_{283.5} - A^\infty_{283.5}} \tag{3}$$

Here, $A^0_{283.5}$ is the sum of the absorbances of completely base-paired poly(A)·poly(U) and free poly(U) with $[(A\cdot U)]/[U] = 1$ and $A^\infty_{283.5}$ is the absorbance of completely base-paired poly(A)·2poly(U). The measured absorbance changes at this wavelength are very small. Thus the accuracy of the $\xi_{(A\cdot U)}$ values derived from Eq. (3) is very low. There is, however, an alternative procedure to determine $\xi_{(A\cdot U)}$. Mass conservation requires that

$$[(A \cdot U)] + 2[(U \cdot A \cdot U)] + [U] = [U^0] \tag{4}$$

where [U] is the concentration of unpaired (U) residues. With the definitions Eqs. (1)–(3) it is readily seen that

$$\xi_{(A\cdot U)} = 2(\xi_{(U)} - \xi_{(U\cdot A\cdot U)}) \tag{5}$$

Thus, the fraction of (A·U) base pairs can be calculated from the absorbances at 260 and 280 nm.

RESULTS

A typical example for the kinetics of the poly(A)·2poly(U) formation after mixing poly(A) with 2poly(U), is shown in Figure 1. It is seen that the absorbance changes reach an apparently time-independent value only after

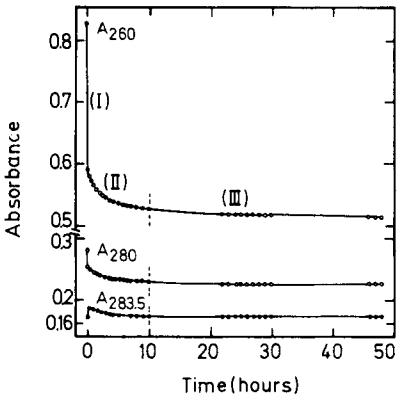

Fig. 1. Time course of the absorbance changes, A_λ, at λ = 260, 280, and 283.5 nm, respectively, after mixing poly(A) and 2poly(U) in 0.15 *M* (Na^+), at pH 7 and 30°C. (I) Rapid phase. (II) and (III) Slow phases.

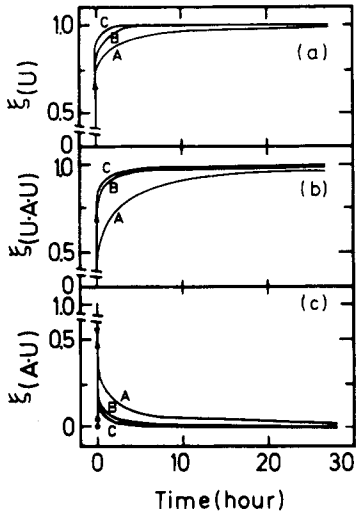

Fig. 2. Extent of structural changes, ξ, as a function of time, calculated according to Eqs. (1)–(5). (a) Incorporation of (U) residues in base pairs. (b) and (c) Formation of (U·A·U) and (A·U) base pairs, respectively. (A) 0.15 *M* Na^+. (B) 0.25 *M* Na^+. (C) 0.35 *M* Na^+. *T* = 30°C.

about 50 hr under the given conditions. It is noted, that in contradistinction to the absorbances A_{260} and A_{280}, the absorbance at 283.5 nm first is larger than $A^0_{283.5}$ for the sum of the separated polymers and then decreases slowly, similar to A_{280} and A_{260}. This feature indicates that after a rapid appearance of (A·U) sequences there is a slower disappearance of these intermediates.

The measured absorbance changes are used to calculate the fractions ξ_U, $\xi_{(A\cdot U)}$, and $\xi_{(U\cdot A\cdot U)}$. These fractions are plotted as a function of time

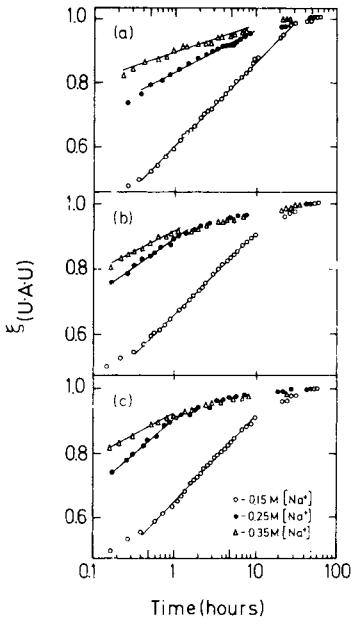

Fig. 3. Extent of (U·A·U) base-pair formation as a function of the logarithm of time, after mixing poly(A) and 2poly(U) (see text), (a) at 10°C (b) at 20°C (c) at 30°C, and at various Na^+ concentrations.

in Figure 2. It is found that within the slow phase (from the minute range on) at any time t, the relationship

$$\xi_{(U\cdot A\cdot U)} = 2\xi_{(U)} - 1 \tag{6}$$

is fulfilled. Equation (6) results, however, from Eq. (5) provided that $\xi_{(A\cdot U)} + \xi_{(U\cdot A\cdot U)} = 1$. The experimental reproduction of Eq. (6) thus suggests that the number of unpaired (A) residues always is negligibly small and that the enlargement of (U·A·U) triple helices occurs at the cost of the (A·U) sequences and unpaired (U) residues. Therefore, the whole slow phase of the triple-helix formation is described by the net reaction

$$(A\cdot U) + U = (U\cdot A\cdot U) \tag{7}$$

In Figure 3, the extent of (U·A·U) formation is plotted as a function of the logarithm of time. It is seen that over large time intervals the relationship between $\xi_{(U\cdot A\cdot U)}$ and $\log t$ is linear. The proportionality factor of this linear relation increases with both increasing salt concentration and increasing temperature. This means that both high-ionic strength and increased temperature facilitate the processes that are rate limiting in the reorganization toward a maximum number of (U·A·U) base pairs.

DISCUSSION

For the following discussion it is convenient to operationally subdivide the time course of the measured absorbance changes into three phases. As

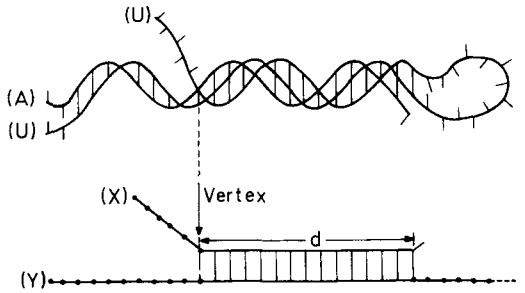

Fig. 4. Scheme of mismatched configuration: hairpin loop of one poly(U) strand base pairing with a poly(A) strand. Bottom: model of unravelling, strand(X) dissociates from strand(Y) (see text).

indicated in Figure 1, a first phase (I) shows rapid absorbance changes lasting up to a few minutes. Then a slower phase (II) follows and lasts several hours. The third phase (III) is extremely slow, lasting up to several days; this last phase contributes, however, only to a small extent to the total visible absorbance changes.

The kinetics of the rapid phase (I) has been intensively studied with the technique of stopped flow.[3,4,12] Chemical relaxation spectrometry has revealed that the elementary steps of (A·U) base-pair formation and dissociation are very rapid (microsecond range) such that even longer oligomers recombine and dissociate in the millisecond range.[13,12,14,15] Consequently, there is no doubt that the rate-limiting processes of the slow phases II and III (in Fig. 1) must be more complicated than simple base pairing.

The observed absorbance changes, after mixing the polymers, suggest that rapid chain-association in phase I leads to complexes in which the base pairs are not completely in register. Apparently the "accumulation" of base pairs in double- and triple-helical sequences is more rapid than the ordered perfect "crystallization" of the entire chain molecules, having all base residues completely matched in base pairs. Particularly, the observed incomplete incorporation of (U) residues into base pairs may reflect "errors" during the rapid chain-associations. These defects may comprise entanglements between single and multistranded chains, hairpin loops due to folding back of parts of the poly(U) strand onto preformed (A·U) stretches (see Fig. 4), or more complicated knots in the chains. Entanglements, knots, and hairpin loops are then "fixed" between multistranded stretches of the macromolecules. When the external conditions of pH, ionic strength, and temperature favor complete base pairing in (U·A·U) sequences, the errors could be very long-lived (metastable) and anneal very slowly.[16,17]

Hydrogen-exchange studies brought evidence that DNA and similar structures are subject to local structural fluctuations.[18,19] This "conformational breathing" of base-paired regions and local helix–coil transitions are the dynamic basis for chain reorganizations of mismatched multichain complexes.

The slow phases of chain-reorganization processes have so far been discussed only on qualitative levels. Among the models proposed for annealing of mismatched configurations are chain slippage or sliding (see, e.g., Ref. 20) or diffusion of single-strand loop along the chains (see, e.g., Eigen, National Colloid Symposium, Univ. of Wisconsin, 1966 and Ref. 21). Rapid diffusion of small loops was proposed to account for kinetic data in oligonucleotide complexes.[15] The slower processes observed in hydrogen-exchange studies of double helices have been interpreted in terms of looped-out single-stranded base stacks propagating by rotation of the whole loop region.[22] Possibly several different mechanisms may contribute to the overall annealing toward more perfect structures with maximum numbers of base pairs. The central question, however, is what is the rate-limiting process in the annealing period? The slowness of annealing would suggest that there are structural defects which require complete unravelling of multistranded regions adjacent to entanglements, knots, and hairpin loops. Indeed, a model for the reorganization of mismatched configurations, in which unravelling is rate limiting, predicts a logarithmic time dependence for the extent of (U·A·U) formation in the slow phase.

THE UNRAVELLING MODEL

In more detail, unravelling is defined as a process in which a strand of a terminal sequence of base pairs unwinds from one end and then starts the formation of base pairs all over again, not necessarily with the previous strand as a partner. If this mechanism leads to an increase in the average number of (U·A·U) segments, the rate-determining step is the unravelling itself, since the formation of "new" bonds is rapid under conditions where the structure with bonded strands is more stable than the free strands. Such an unravelling model has been quantitatively elaborated by Julius Jackson (Jackson and Silberberg, in preparation*). The unravelling process is schematically represented in Figure 4, where a more flexible chain X, dissociates from a more rigid strand Y. At time t, the strands are connected by d bonds. The question to be asked is what is the time it will take for the Brownian motion to bring the vertex a number of d bonds to the right, as to permit complete separation of the chains and subsequent recombination with more connections than previously. This problem can be solved by applying a first transit-time treatment.[23,24] The time required to displace the vertex by d bonds is given by

$$t = \frac{\tau}{2(p-q)^2} \cdot \exp\left[2(p-q)d\right] \tag{8}$$

where p is the probability for closing one bond, q is the probability for opening one bond, and τ is the average time constant for the closing and

* The publication treating the theory of the unravelling model (as well as several alternatives such as chain sliding and loop diffusion) has been delayed because of the sudden death of J. Jackson. For the benefit of the reader, we briefly give the main features of the theory.

opening process. The total number of bonds, $B(t)$, in the mixture at a time t is given by the following expression:

$$B(t) = B(t_{\min}) + \frac{d_0 \cdot k(t_{\min})}{2(p-q) \cdot (d_{\max} - d_{\min})} \ln \frac{t}{t_{\min}} \tag{9}$$

where $B(t_{\min})$ is the number of bonds at a reference time $t_{\min}$, $k(t_{\min})$ is the number of knots at $t_{\min}$, d is the number of (new) bonds formed after each unravelling event, $d_{\max}$ is the maximum number of bonds between the two strands, and $d_{\min}$ is the number of bonds at the reference time $t_{\min}$. We see in Eq. (9), that an annealing process in which the rate-determining step is the unravelling results in a logarithmic dependence on time of the number of bonds.

In the slow annealing phase of the poly(A)·2poly(U) formation, the resolution of mismatched configurations requires partial helix–coil transition (unravelling) of increasingly longer (U·A·U) stretches. Applying the formalism of the unravelling model, briefly presented above, to the case of annealing mismatched (U·A·U) stretches, we may consider as X the more flexible stretches of (U) residues and as Y the more rigid stretches of (A·U) residues; see Figure 4. We denote by $d_{\min}$ the number of (U·A·U) base pairs at $t_{\min}$, by $d(t)$ the number of (U·A·U) bonds present at time t, by $d_{\max}$ the maximum number of (U·A·U) bonds between the strands, and by $t_{\max}$ the time interval required for recombining $d_{\max}$ bonds. At the reference time $t_{\min}$, $k(t_{\min})$ knots must be resolved. The number of (U·A·U) bonds, $B(t_{\max})$, formed at the end of this annealing process, is equal to the sum of the number of (U·A·U) bonds at $t_{\min}$, $B(t_{\min})$, and the number of bonds formed after resolving of the knots. Thus

$$B(t_{\max}) - B(t_{\min}) = k(t_{\min}) \cdot d_0 \tag{10}$$

Introducing Eq. (10) into Eq. (9) one obtains

$$B(t) = B(t_{\min}) + \frac{B(t_{\max}) - B(t_{\min})}{2(p-q) \cdot (d_{\max} - d_{\min})} \ln \frac{t}{t_{\min}} . \tag{11}$$

We now may transform Eq. (11) so as to be expressed in terms of the extent of (U·A·U) formation, $\xi_{(U\cdot A\cdot U)}(t)$. The number of (U·A·U) bonds, $B(t)$, is proportional to $\xi_{(U\cdot A\cdot U)}(t)$, since $B(t) = N \cdot \xi_{(U\cdot A\cdot U)}(t)$, where N is the total number of possible (U·A·U) base pairs. The number of bonds $d(t)$, in one macromolecular complex, is also proportional to $\xi_{(U\cdot A\cdot U)}(t)$, since $d(t) = n \cdot \xi_{(U\cdot A\cdot U)}(t)$, where n is the number of (U·A·U) base pairs. Introducing these proportionalities into Eq. (11), the relationship

$$\xi_{(U\cdot A\cdot U)}(t) = \xi_{(U\cdot A\cdot U)}(t_{\min}) + \frac{1}{2(p-q)n} \ln \frac{t}{t_{\min}} \tag{12}$$

is obtained. From Eq. (12) it is seen that the application of Jackson's unravelling model to our case leads to a logarithmic dependence on time of the extent of (U·A·U) segment formation. The data presented in Figure

TABLE I
Values for the Probability, p, of Formation of a (U·A·U) Base Triple and for the Probability, q, of dissociating a (U·A·U) Segment[a]

Temperature, °C	Na^+ Concentration, M	p	q
10	0.15	0.522	0.478
	0.25	0.544	0.456
	0.35	0.557	0.443
20	0.15	0.522	0.478
	0.25	0.533	0.467
	0.35	0.546	0.454
30	0.15	0.521	0.479
	0.25	0.525	0.475
	0.35	0.542	0.458

[a] Calculated from the experimental results of Figure 3, using the formalism of the unravelling model, for the time where $n = 100$.

3 are analyzed in terms of Eq. (12). The values of p and q for the time where $n = 100$ are listed in Table I. It is thermodynamically consistent that the probability p of closing a bond increases both with increasing ionic strength and decreasing temperature.

In summary, the kinetic data show that the slow part of the poly(A)·2poly(U) formation involves steps in which the number of (U·A·U) base pairs increases at the cost of (A·U) and (U) stretches. It is this overall chain reorganization that characterizes the "annealing" period. Among several quantitative models proposed by Jackson for the annealing process, the unravelling mechanism predicts a kinetic behavior consistent with the experimental data. Thus mismatched configurations of this polynucleotide system reorganize by completely resolving base-paired sequences before new multistranded regions with more base pairs are formed. A recent kinetic theory for the relaxation behavior of triple-stranded helix–coil transitions also involves complete uncoiling; this theory correctly describes kinetic data of triple-helical collagen fragments.[25] Inspection of recent data on the refolding of crab satellite DNA and analogs[26] shows that parts of the refolding process are linearly dependent on the logarithm of time. Here, too, unravelling appears to be the rate-limiting process in the annealing period.

The kinetic analysis of the poly(A)–poly(U) system shows that the rate of annealing increases at elevated temperatures and increased ionic strength. If now unravelling generally is the rate-determining step in the annealing period of multistranded polynucleotide complexes, repetitive heating and slow cooling (tempering) of the reaction mixture should minimize long-lived defects and lead to an optimum number of base pairs.

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