Kinetics of Binding of Single-Stranded DNA Binding Protein from *Escherichia coli* to Single-Stranded Nucleic Acids†

Claus Urbanke* and Achim Schaper

Medizinische Hochschule, Zentrum Biochemie, Abteilung Biophysikalische Chemie, Konstanty-Gutschow Strasse 8, D-3000 Hannover 61, FRG

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ABSTRACT: The time course of the reaction of *Escherichia coli* single-stranded DNA binding protein (*E. coli* SSB) with poly(dT) and M13mp8 single-stranded DNA has been measured by fluorescence stopped-flow experiments. For poly(dT), the fluorescence traces follow simple bimolecular behavior up to 80% saturation of the polymer with *E. coli* SSB. A mechanistic explanation of this binding behavior can be given as follows: (1) *E. coli* SSB is able to translocate very rapidly on the polymer, forming cooperative clusters. (2) In the rate-limiting step of the association reaction, *E. coli* SSB is bound to the polymer only by one or two of its four contact sites. As compared to poly(dT), association to single-stranded M13mp8 phage DNA is slower by at least 2 orders of magnitude. We attribute this finding to the presence of secondary structure elements (double-stranded structures) in the natural single-stranded DNA. These structures cannot be broken by *E. coli* SSB in a fast reaction. In order to fulfill its physiological function in reasonable time, *E. coli* SSB must bind newly formed single-stranded DNA immediately. The protein can, however, bind to such pieces of the newly formed single-stranded DNA which are too short to cover all four binding sites of the *E. coli* SSB tetramer.

The single-stranded DNA (ssDNA) binding protein of *Escherichia coli* (*E. coli* SSB) plays a vital role for all processes of the bacterial cell involving the metabolism of DNA. The native protein is a tetramer of four identical subunits arranged in a D_2 symmetry (Ollis et al., 1983) with a total molecular weight of 75 500 (Sancar et al., 1981). It binds strongly and cooperatively to ssDNA with no sequence preferences on natural nucleic acids. Binding to double-stranded DNA has not been observed (Chase & Williams, 1986).

For the physiological function of *E. coli* SSB, it is generally assumed that the protein binds to every ssDNA produced during the life cycle of the cell. The ssDNA is wound around the *E. coli* SSB, making contacts to four binding sites on the tetramer (Krauss et al., 1981). In this structure, the single-stranded regions are protected from enzymatic degradation (Chrysogelous & Griffith, 1982) and the formation of intramolecular helices (Reckmann et al., 1985).

However, *E. coli* SSB bound strongly to the ssDNA should not interfere with processes of the DNA metabolism like, e.g., the polymerase reaction during replication and repair or strand exchange in recA-catalyzed recombination. Even more, all these processes are greatly enhanced by bound *E. coli* SSB; the very stable complex with ssDNA must therefore be considered as a dynamic structure.

Thermodynamic parameters for the binding of *E. coli* SSB to single-stranded nucleic acids have been investigated in the last years in great detail. For a review, see Chase and Williams (1986) and Greipel et al. (1989). Under physiological conditions, the binding constants for the interaction of *E. coli* SSB with poly(dT) and native ssDNAs were found to be higher than 10^{10} M^{-1}. This strong binding reflects an extremely long lifetime of the complex which is of the order of several minutes (Schneider & Wetmur, 1982) and which apparently contradicts the physiological role of *E. coli* SSB in catalyzing metabolic processes at the single-stranded sites of the DNA.

The binding of *E. coli* SSB to ssDNA is accompanied by a drastic fluorescence quench of 50–90% and can therefore be observed easily. Since the quench is independent of the degree of saturation of the polymer with *E. coli* SSB (Lohman & Overman, 1985), fluorescence measurements can be used directly to determine the fractions of free and bound *E. coli* SSB. The length of the polymer covered by a single *E. coli* SSB tetramer depends on salt conditions and on the structure of the ssDNA used. Below 0.02 M NaCl, *E. coli* SSB bound to poly(dT) covers 33 nucleotides per tetramer. Above 0.3 M NaCl, this value increases to 63 (Bujalowski & Lohman, 1986). For native ssDNA, the stoichiometries observed are somewhat larger, reflecting the presence of stable secondary base-paired structures inaccessible to *E. coli* SSB (Bujalowski et al., 1988).

The binding isotherms can be described by models for the cooperative binding of multidentate ligand to a linear polymer of equivalent subunits (McGhee & VonHippel, 1974; Schwarz & Watanabe, 1983) either with unlimited or with limited cooperativity (Bujalowski & Lohman, 1987). The binding constant depends on the substrate used and increases in the order poly(dA) = poly(rU) < single-stranded phage DNA (ϕX174,M13mp8) = poly(dT).

Stopped-flow kinetics of the interaction of *E. coli* SSB with homopolynucleotides showed that the association rate is nearly diffusion-controlled (Lohman, 1986; Römer et al., 1984). However, these kinetic studies dealt only with the association in the limit of low binding density and therefore used a high excess of ssDNA over *E. coli* SSB.

Statistical theory for the binding of multidentate ligands to a linear polymer (see above) predicts that at increasing degrees of saturation potential binding sites for the ligand are hidden by ligands bound less than the length of a single binding site apart. This effect should lead to an apparent anticooperativity with a decreasing apparent bimolecular association rate constant at higher binding densities.

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* To whom correspondence should be addressed.

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In our study, we will investigate the association kinetics for the binding of *E. coli* SSB to poly(dT) and to native as well as denatured single-stranded M13mp8 DNA by stopped-flow measurements. To deal with the association kinetics at higher binding densities, it will be necessary to solve the bimolecular rate equation taking into account the apparent anticooperativity as described by the statistical theory. We will show how this association mechanism depends on the degree of saturation and structure of the ssDNA. Physiological implications of the results will be described.

Materials and Methods

The standard buffer used in this study contains 0.02 M potassium phosphate, pH 7.2, 0.1 mM EDTA, and the indicated amount of NaCl.

Poly(dT) was purchased from Pharmacia, Freiburg. It had a length of ca. 1400 nucleotides as judged from polyacrylamide gels and analytical ultracentrifugation. All experiments presented in this paper were done with the same charge of poly(dT). Differences in the kinetic curves between two different charges could not be observed.

M13mp8 ssDNA was isolated from a phage suspension grown on *E. coli* SSB strain JM103 by the method of Yamamoto et al. (1970). Denaturation of remaining secondary structures was achieved by treatment with 1 M glyoxal at 50 °C in the standard buffer containing 50% (v/v) dimethyl sulfoxide for 1 h as described by Carmichael and McMaster (1980).

Concentrations of polynucleotides were determined by UV absorption using an extinction coefficient at maximum of 8600 M^{-1} cm^{-1} for a single base in poly(dT) and 7370 M^{-1} cm^{-1} per base residue in M13mp8 at 0.1 M NaCl (Berkowitz & Day, 1974).

E. coli SSB was isolated from an overproducing strain (Bayer et al., 1989) by the method of Lohman et al. (1986a). It was always at least 98% pure as judged from polyacrylamide gels. Concentrations were determined by UV absorption using an extinction coefficient of 113 000 M^{-1} cm^{-1} at 280 nm for the *E. coli* SSB tetramer. This extinction coefficient was determined by extensive dialysis of *E. coli* SSB against a buffer containing 0.5 M ammonium carbonate, by drying the protein thoroughly at 110 °C, and by weighing. The extinction coefficient determined this way agrees with values obtained by amino acid analysis (Williams et al., 1983; Lohman & Overman, 1985) or protein determinations with colorimetric methods (Ruyechan & Wetmur, 1976). However, it is larger than the coefficient measured by the refractive index method of Babul and Stellwagen (1969) as used by us before (Bayer et al., 1989). We have reproduced the refractive index measurements several times and could not find any reason for this discrepancy.

Stopped-Flow Experiments. Stopped-flow measurements were performed in a modified version of the Durrum–Gibson stopped-flow apparatus. Protein fluorescence was excited at 290 nm, and emision was monitored through a combination of Schott UG11 and Schott KV309 filters. Fluorescence was recorded by an IBM compatible personal computer equipped with a DiSys PCI analog/digital input/output card. The acquisition program allowed for a continuous increase in time intervals between two measurements, and, therefore, reactions from milliseconds up to 20 min could be recorded in a single experiment. For noise reduction, experiments were usually repeated 3–10 times and then added upon each other. Since a single fluorescence trace contains information from the millisecond to the minute range, we decided to present the stopped-flow traces on a logarithmic rather than on a linear time scale (Walmsley & Bagshaw, 1989).

Theory

The reaction of *E. coli* SSB with polynucleotides can be described in a very simplified manner by

$$E.\ coli\ \text{SSB} + \text{poly(dT)} \xrightarrow{k_1} E.\ coli\ \text{SSB·poly(dT)}$$

where k_1 is the rate constant for association. A mechanism with a single association rate constant implies that effects of cooperativity do not influence the rate-limiting step. Due to the high stability of the *E. coli* SSB·poly(dT) complex ($K_{assoc} > 10^{10}$ M^{-1}) [cf. Greipel et al. (1989)], dissociation can be neglected at the concentrations used in this work. To satisfy the stoichiometry, the polymer concentration must be given as the concentration of available binding sites, i.e., the length of the polymer covered by the protein, n. Given the total concentration of polymer in nucleotides, c_p, the concentration of binding sites will be $c_s = c_p/n$. The phenomenological rate equation is then given by

$$dc_{SSB}/dt = -k_1 c_{SSB} c_s \tag{1}$$

where c_{SSB} is the concentration of free *E. coli* SSB at any time of the reaction. Since c_s likewise denotes the concentration of free and available binding sites, this equation will only hold as long as bound *E. coli* SSB does not create gaps of free nucleotides shorter than the binding site length n. Under these conditions, the rate equation can be integrated and linearized to give

$$t = \frac{1}{k_1(c^0_{SSB} - c^0_s)} \ln\left[\frac{1 + (c^0_{SSB} - c^0_s)/(c^0_s - c^b_{SSB})}{1 + (c^0_{SSB} - c^0_s)/c^0_s}\right] \tag{2}$$

where the superscript 0 denotes initial concentrations and c^b_{SSB} is the concentration of bound *E. coli* SSB at any time. At higher binding densities, the formation of short gaps should lead to an apparent decrease in the association rate constant k_1.

For the thermodynamic equilibrium between a multidentate ligand and a linear polymer with equivalent binding sites, Schwarz and Watanabe (1983) calculated the distribution of free gaps as a function of saturation. Neglecting end effects, they give for the concentration of a free gap of i nucleotides c^i_s

$$c^i_s = \frac{c_p(\lambda - 1)\theta}{n[1 + q(\lambda - 1)]k^i} \tag{3}$$

with

$$\lambda = 1 + \{[(1 - \beta)^2 + 4q\beta]^{1/2} - (1 - \beta)\}/2q \tag{4}$$

and

$$\beta = \frac{\theta/n}{1 - \theta} \tag{5}$$

where θ is the fraction of the polymer complexed with *E. coli* SSB and q is the cooperativity parameter defined as the ratio of the binding affinity of an isolated *E. coli* SSB to the binding affinity of an *E. coli* SSB bound next to another one. For a simplified kinetic approach, we now have to calculate the concentration of potential association sites on the polymer as a function of time and saturation. In our mathematical model, we assume that the distribution of *E. coli* SSB on the polymer is always identical with the equilibrium distribution and therefore time-independent. This assumption implies that the

rate-limiting step is the bimolecular association and that any reorganization of the complex is fast. Let us now assume that *E. coli* SSB covers r nucleotides in the rate-limiting step where r need not be equal to n. Any gap of free nucleotides m residues long with $m \geq r$ then constitutes $m - r + 1$ potential association sites, and the fraction of all of these sites as compared to the total number of polymer residues is given by a summation over all gaps:

$$\Phi = \frac{(\lambda - 1)\theta}{n[1 + q(\lambda - 1)]} \sum_{i=r}^{\infty}(i - r + 1)\lambda^{-i} \quad (6)$$

For λ not much larger than 1, the calculation of the sum can replaced by an integration:

$$\sum_{i=r}^{\infty}(i - r + 1)\lambda^{-i} = \sum_{r}^{\infty}(i - r + 1)\exp(-i \ln \lambda)\, di \quad (7)$$

to give

$$\Phi = \frac{(\lambda - 1)\theta(1 + \ln \lambda)}{n[1 + q(\lambda - 1)]k^r (\ln \lambda)^2} \quad (8)$$

For application to eq 1, the fraction of polymer residues available for binding relative to the fraction of free residues is given by

$$\Phi' = \frac{\Phi}{1 - \theta} \quad (9)$$

and the rate equation is given as

$$dc_{SSB}/dt = -k_1\Phi'(c^0_{SSB} - c^b_{SSB})(c^0_s - c^b_{SSB}) \quad (10)$$

In the limit $\theta \rightarrow 0$ or for the case of infinite cooperativity, Φ' will be unity as can be shown by setting $\ln \lambda \approx \lambda - 1$ and application of L'Hospitals rule.

Evaluation of Stopped-Flow Experiments. For the evaluation of the stopped-flow experiments, we used a nonlinear least-squares fitting algorithm (Powell, 1965). In this procedure, eq 10 was integrated by a multistep predictor corrector method (Gear, 1971), and the fluorescence increments for free and bound *E. coli* SSB as well as the cooperativity q, the length of the initial association site r, and the association rate constant k_1 for the best fit were calculated. The algorithm allows for keeping some of the parameters constant while varying others to reduce ambiguities in the fitting results. Several stopped-flow experiments at different concentrations were used simultaneously in the fitting procedure to give a single set of parameters.

For small concentration changes in the approach to equilibrium, eq 1 can be linearized and solved for k_1 as in a relaxation experiment, giving an exponential decay with a time constant of

$$\tau^{-1} = k_1(\bar{c}_{SSB} + \bar{c}_s) \quad (11)$$

where $\bar{c}_{SSB}$ and $\bar{c}_s$ denote the final equilibrium concentrations of *E. coli* SSB and poly(dT), respectively. Since all experiments of this kind were performed with an excess of poly(dT) over *E. coli* SSB, $\bar{c}_{SSB}$ can be neglected due to the large binding constant.

Results and Discussion

The fluorescence of *E. coli* SSB decreases up to 90% when bound to single-stranded polynucleotides. This effect can be used to monitor the binding of the protein to poly(dT) in kinetic experiments. Figure 1 shows a fluorescence trace of a typical stopped-flow experiment for the reaction of an excess of *E. coli* SSB with poly(dT) at 0.3 M NaCl. At this salt concentration, 63 nucleotides are covered by an *E. coli* SSB

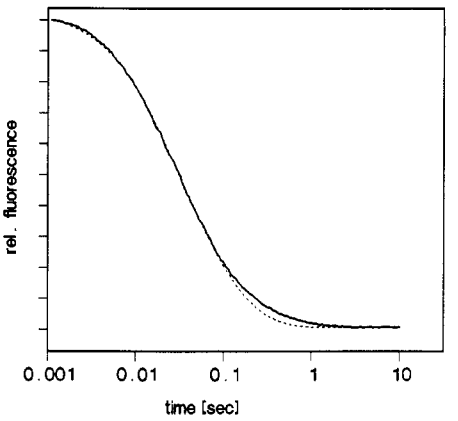

FIGURE 1: Fluorescence stopped-flow trace for the reaction of 0.34 μM *E. coli* SSB (tetramer) with 17.6 μM poly(dT) (0.28 μM binding sites) at 0.3 M NaCl in the standard buffer. The dashed line represents the course of reaction as calculated for a simple bimolecular mechanism according to eq 2.

tetramer (Bujalowski & Lohman, 1986). As shown in Figure 1, the theoretical curve according to eq 2 for a simple bimolecular association mechanism without any correction for an apparent kinetic anticooperativity using $k_1 = 2 \times 10^8$ M^{-1} s^{-1} describes well the measurement in a range from 0% up to 80% saturation. This suggests that during the course of association cooperative clusters of bound *E. coli* SSB are formed which reduce the number and increase the length of the gaps on the polymer.

To test whether the distribution of *E. coli* SSB on poly(dT) during the course of association always follows an equilibrium distribution, a complex of *E. coli* SSB with poly(dT) was allowed to reach equilibrium. It was then mixed with a small aliquot of further *E. coli* SSB in the stopped-flow instrument. Under otherwise identical concentrations and the same final degrees of saturation of poly(dT) with *E. coli* SSB, both components were directly mixed in the stopped-flow instrument. In both cases, the approach to equilibrium can be analyzed as in a relaxation experiment (eq 11).

As shown in Figure 2, the relaxation times for a transiently formed complex and for the preformed complex are identical. This demonstrates that the equilibrium distribution of bound *E. coli* SSB on poly(dT) is reached immediately after the association event. No significant deviation from the bimolecular behavior can be observed, and the same k_1 as in Figure 1 is evaluated.

Thus, the pathway for the reaction of *E. coli* SSB with poly(dT) can be described as follows: *E. coli* SSB is bound to the poly(dT) and immediately forms cooperative clusters with those proteins already present on the polymer. The rate-limiting step is the association of protein and nucleic acid, and the rearrangement of the complex is fast. In this mechanism, transiently formed short gaps will enlarge quickly by translocation of bound *E. coli* SSB on the polymer to give clusters of cooperatively bound proteins.

A rapid translocation has already been deduced from NMR measurements of poly(dT)·*E. coli* SSB complexes (Römer et al., 1984). The mechanism of this translocation involves a partial dissociation of the DNA from one of the contact sites and a reassociation of a different part of the polymer. Any other mechanism with a slow rearrangement must at least transiently form a distribution of *E. coli* SSB corresponding to an equilibrium distribution for the noncooperative binding where according to eq 9 at approximately 30% saturation more than half of the free nucleotides are located in gaps too short to accommodate additional *E. coli* SSB. A mechanism in

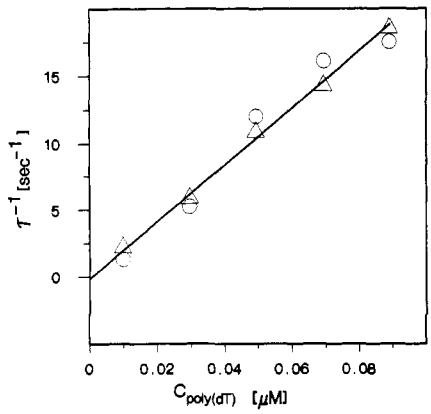

FIGURE 2: "Relaxation" kinetics for the binding of *E. coli* SSB to presaturated poly(dT) at 0.3 M NaCl in the standard buffer. The relaxation time is plotted vs the final free concentration of poly(dT) given in binding sites ($n = 63$). (O) 12.6 μM poly(dT) (0.2 μM binding sites) was incubated with 0, 0.04, 0.08, 0.12, and 0.16 μM *E. coli* SSB (0, 20%, 40%, 60%, and 80% saturation) for 1 h. This preformed complex was then mixed in the stopped-flow instrument with 0.02 μM *E. coli* SSB, thereby raising the saturation a further 10%. Note that final concentrations are lower by a factor 2 resulting from the dilution in the stopped-flow instrument. (Δ) 12.6 μM poly(dT) (0.2 μM binding sites) was mixed in the stopped flow with 0.02, 0.06, 0.10, 0.14, and 0.18 μM *E. coli* SSB. Only the last part of the fluorescence trace corresponding to the reaction of 0.01 μM *E. coli* SSB were evaluated to give the relaxation time.

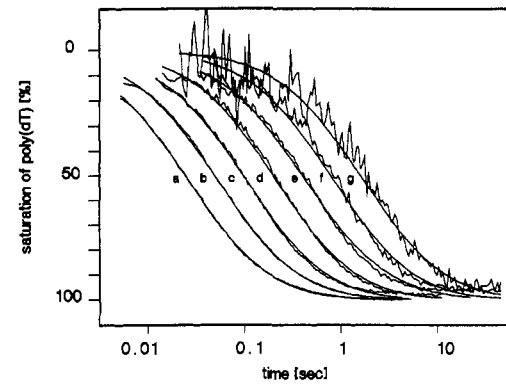

FIGURE 3: Concentration dependence of the reaction of *E. coli* SSB with poly(dT) at 0.3 M NaCl in the standard buffer. The smooth lines give the best fit according to eq 10 with $q = 150$, $k_1 = (2.5 \pm 0.1) \times 10^8$ M^{-1} s^{-1}, and $r = 15 \pm 4$. Concentrations were (a) 0.34 μM *E. coli* SSB mixed with 17.6 μM poly(dT) (0.28 μM binding sites), (b) 0.17 μM *E. coli* SSB mixed with 8.8 μM poly(dT) (0.14 μM binding sites), (c) 0.085 μM *E. coli* SSB mixed with 4.4 μM poly(dT) (0.07 μM binding sites), (d) 0.042 μM *E. coli* SSB mixed with 2.2 μM poly(dT) (0.035 μM binding sites), (e) 0.021 μM *E. coli* SSB mixed with 1.1 μM poly(dT) (0.017 μM binding sites), (f) 0.011 μM *E. coli* SSB mixed with 0.55 μM poly(dT) (0.0083 μM binding sites), (g) 0.005 μM *E. coli* SSB mixed with 0.28 μM poly(dT) (0.0044 μM binding sites). Note that the curves are normalized to the degree of saturation of poly(dT) with *E. coli* SSB and that the S/N ratio decreases drastically with decreasing concentration.

which the rearrangement is reached via free *E. coli* SSB can be ruled out because of the extremely slow dissociation rate constant (0.1–0.01 s^{-1}) (Schneider & Wetmur, 1982).

The reaction mechanism involving a fast rearrangement of bound *E. coli* SSB can be described by eq 10 setting the length of the polymer covered in the rate-limiting association step, r, equal to the number of nucleotides covered at equilibrium. A fit of the association rate constant k_1 and the cooperativity parameter q yields $k_1 = (2.5 \pm 0.1) \times 10^8$ M^{-1} s^{-1} and $q = 2500 \pm 500$. This cooperativity parameter is far too large to be compatible with any measurements reported so far. From electron micrographs of the *E. coli* SSB·poly(dT) complex, Greipel et al. (1987) deduce a cooperativity constant not larger than 150 under the same buffer conditions as used in our stopped-flow measurements. Lohman et al. (1986b) report cooperativity parameters of the order of 50 for several other polynucleotides. Dissociation of *E. coli* SSB from poly(dT) occurs in a first-order reaction (Bayer et al., 1989) whereas a cooperativity constant of more than 1000 would require this dissociation to be of zeroth order (Lohman, 1983). With cooperativity parameters of 150, the mean length of a cooperative cluster of bound *E. coli* SSB on poly(dT) of infinite length at 80% polymer saturation is 3–4 (Schwarz & Watanabe, 1983), and the influence of the finite length of the poly(dT) used (approximately 22 binding sites for *E. coli* SSB) can be neglected to within an error of 10% (Epstein, 1978).

A second mechanistic feature facilitating the association even to highly saturated poly(dT) can be derived from binding studies with oligonucleotides. It could be shown that the *E. coli* SSB tetramer at 0.3 M NaCl contains four contact sites for the binding of ssDNA (Krauss et al., 1981). These contact sites act highly cooperatively, and *E. coli* SSB associated to polymeric nucleic acid with less than all four of them involved could not be observed. However, it might be sufficient that in an initial association event only one or two of these contact sites bind to the nucleic acid with the remaining ones following in a rapid intramolecular reorganization.

Therefore, we assume that in the rate-limiting step for the association *E. coli* SSB has not yet covered all of the 63 nucleotides as in the final equilibrium state. Figure 3 shows stopped-flow traces of an experiment with excess *E. coli* SSB over poly(dT) at several very different concentrations of both reactants. All experiments can be simultaneously fitted by using a cooperativity q = 150, giving the same association rate constant, $k_1 = (2.5 \pm 0.1) \times 10^8$ M^{-1} s^{-1}. Taking into account the length of the poly(dT) used (1400 nucleotides), this value is of the same order as earlier data (Lohman, 1986; Römer et al., 1984); it indicates a diffusion-controlled association mechanism. However, the number of nucleotides covered in the rate-limiting step must be reduced to $r = 15 \pm 4$ to achieve a satisfactory agreement between measured and theoretical curves. Nearly diffusion-controlled association rates are also observed for short oligo(dT) molecules accommodating not more than one *E. coli* SSB tetramer (Krauss et al., 1981; own data not shown). Therefore, the association rate of a singly bound *E. coli* SSB on the polymer is fast, and cooperativity effects influence the lifetime of the complex rather than the association rate constant. This again rules out a cooperative association mechanism, in which the association of the first *E. coli* SSB is very slow compared to the growing of a cooperative cluster.

From the arguments outlined above, we conclude that the initial association of only one or two binding sites of a free *E. coli* SSB is sufficiently stable and that rapid reshuffling of bound proteins leads to the association of the remaining binding sites. A similar model with a partial association has been proposed by Lohman et al. (1986b) where the dependence of the length of poly(dT) covered by *E. coli* SSB, n, on the salt concentration has been attributed to the association of the protein via less than all four binding sites. Our kinetic experiments show that this partially associated *E. coli* SSB is the nucleus for full association at high salt concentrations. Figure 4 shows a schematic drawing of this initial association complex.

To further test the physiological relevance of our kinetic measurement with synthetic poly(dT), we decided to use a natural ssDNA, M13mp8 DNA. Figure 5 shows a comparison of the association kinetics of *E. coli* SSB to single-stranded

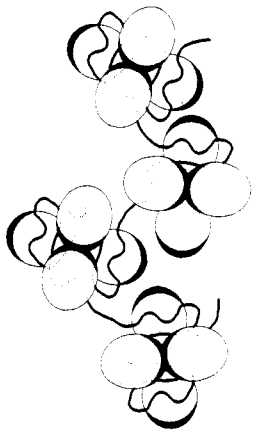

FIGURE 4: Schematic cartoon depicting the nucleation step for the binding of *E. coli* SSB to a partially saturated poly(dT). The gap between the bound *E. coli* SSB tetramers is too short to accommodate full binding by all four contact sites, but this complex is stable and will reach full contact by intramolecular rearrangement.

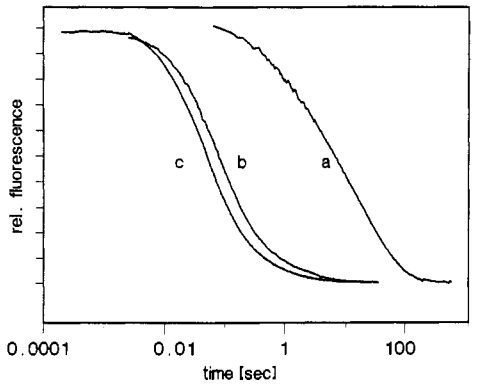

FIGURE 5: Fluorescence stopped-flow traces for the reaction of different polymeric nucleic acids with *E. coli* SSB. (a) 0.18 μM *E. coli* SSB mixed with 14.8 μM M13mp8 ssDNA. (b) 0.26 μM *E. coli* SSB mixed with 13.6 μM M13mp8 ssDNA denatured by treatment with glyoxal as described under Materials and Methods. (c) 0.34 μM *E. coli* SSB mixed with 17.6 μM poly(dT).

M13mp8 DNA, M13mp8 DNA treated with glyoxal, and poly(dT). There is a drastic dependence of the association rates on the nature of the nucleic acids. Poly(dT) (trace c) reacts almost diffusion-controlled, whereas the native M13mp8 DNA (trace a) is more than 2 orders of magnitude slower. In addition, the association of *E. coli* SSB to M13mp8 DNA cannot be described by a simple bimolecular association mechanism. The apparent association rate as evaluated from the slope of the fluorescence trace varies from 4×10^6 M^{-1} s^{-1} for binding the first *E. coli* SSB to 0.8×10^6 M^{-1} s^{-1} at 90% saturation. The drastic decrease of the apparent association rate constant and the strong heterogeneity of the reaction most probably reflect the ubiquitous presence of non-single-stranded regions in M13mp8 DNA. Treatment of the M13mp8 DNA with glyoxal, a reagent that modifies guanosines covalently and thereby blocks formation of GC base pairs (Carmichael & McMaster, 1980), releaves this decrease in the apparent association rate constant to a great extent (Figure 5, trace b).

"Melting" of DNA helices by *E. coli* SSB at room temperature can occur via two different pathways. (1) Immediately after a base pair transiently opens at the end of a helix, *E. coli* SSB binds and thereby "zippers" up the double strand. (2) *E. coli* SSB can only bind to longer stretches of ssDNA, and helices must be disrupted to a greater extent to allow for binding.

These different pathways can be differentiated according to their time scale. The first pathway is used by the *E. coli* DNA polymerase (Klenow fragment). Here it could be shown that the secondary structures present in M13mp8 DNA do not constitute any barriers to the polymerization reaction. For other polymerases, only some of the secondary structures of M13mp8 DNA constitute a barrier (Reckmann et al., 1985). If binding of *E. coli* SSB occurred via this sequential mechanism, it should not be slowed down too much. At least for some part of the M13mp8 DNA this mechanism predicts fast association rates comparable to those to poly(dT). This is clearly not the case, and binding via single, transiently opened, base pairs can be ruled out.

The second mechanism requires longer stretches of ssDNA possibly spanning two or more contact sites on the *E. coli* SSB tetramer. The population of such nucleotide stretches in M13mp8 DNA is rather low (Reckmann et al., 1985). Secondary structure calculations showed that more than 80% of the bases are potentially involved in secondary structures and can thus kinetically be blocked for the reaction with *E. coli* SSB. A single contact site on *E. Coli* SSB interacts with ca. two nucleotides (Schomburg, 1985), much less than needed to span the distance between contact sites. In order to bind at two contact sites, a ssDNA has to be at least 10–15 nucleotides long, and, therefore, any single-stranded stretch shorter than that is of no advantage for the binding reaction.

CONCLUSIONS

E. coli SSB binds to free ssDNA in a fast diffusion-controlled process. The resulting complex undergoes dynamic transformations in which the *E. coli* SSB tetramer translocates on the polymer. The mechanism of this translocation probably involves partial dissociation of ssDNA from one or two of the four polynucleotide binding sites of the protein (Römer et al., 1984). Association of *E. coli* SSB can use a pathway where only one or two of the four binding sites on the tetramer form a stable nucleus. This mechanism overcomes the blocking of potential binding sites by other proteins bound leaving gaps too short to be covered by a single *E. coli* SSB tetramer at equilibrium. The seondary structure of a polynucleotide blocks most of the potential binding sites and thus slows down the process of association extremely. Obviously, *E. coli* SSB is not able to disrupt helices base pair by base pair but must rather wait for a certain stretch of nonpaired bases, a situation that is rare at room temperature. Since there is no initial fast binding process on native ssDNA (M13mp8 phage DNA), we conclude that almost all bases in this ssDNA are involved in some kind of secondary structure. For the function of *E. coli* SSB within the cell, these findings make it necessary that *E. coli* SSB binds to every ssDNA at the moment of its production. Otherwise, secondary structures would be formed preventing *E. coli* SSB from reaching its target in a reasonable time.

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Characterization of the Multiple Catalytic Activities of Tartrate Dehydrogenase†

Peter A. Tipton* and Jack Peisach
Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461
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ABSTRACT: Tartrate dehydrogenase (TDH) has been purified to apparent homogeneity from *Pseudomonas putida* and has been demonstrated to catalyze three different NAD^+-dependent reactions. TDH catalyzes the oxidation of (+)-tartrate to form oxaloglycolate and the oxidative decarboxylation of D-malate to form pyruvate and CO_2. D-Glycerate and CO_2 are formed from *meso*-tartrate in a reaction that is formally a decarboxylation with no net oxidation or reduction. The steady-state kinetics of the first two reactions have been investigated and found to follow primarily ordered mechanisms. The pH dependence of V and V/K was determined and indicates that catalysis requires that a base on the enzyme with a pK of 6.7 be unprotonated. TDH activity requires a divalent and a monovalent cation. Kinetic data suggest that the cations function in substrate binding and facilitation of the decarboxylation of β-ketoacid intermediates.

The study of the stereospecificity of microbial metabolism of tartaric acid has a long history; Duclaux (1920) noted that Pasteur observed that *Penicillium* was able to grow on a racemic solution of ammonium tartrate. As growth progressed, the solution acquired optical activity. After the cessation of growth, the solution contained only levo-rotatory tartrate, demonstrating that the microorganisms were able to utilize only one isomer of tartrate.

More recently, the pathways for utilization of (+)-tartrate, (−)-tartrate, and *meso*-tartrate in *Pseudomonas* have been elucidated, and many of the enzymes involved have been isolated (Clarke & Ornston, 1975). Kohn et al. (1968) reported the isolation and characterization of a tartrate dehydrogenase from a strain of *Pseudomonas putida* which required Mn^{2+} and K^+ for activity and catalyzed the oxidation of both (+)-tartrate and *meso*-tartrate to oxaloglycolate. Because these requirements for inorganic cofactors were relatively novel for a dehydrogenase, we set out to determine their functions in the reported catalytic reactions. We now wish to report that when *P. putida* is grown on a mixture of (+)-tartrate and *meso*-tartrate as the sole carbon sources, an enzyme is produced which catalyzes the conversion of (+)-tartrate to oxaloglycolate, *meso*-tartrate to D-glycerate and CO_2, and D-malate to pyruvate and CO_2. In other words, for these three similar substrates, the same enzyme catalyzes

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