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Conformational Coupling in DNA Polymerase Information Transfer [and Discussion]

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Conformational coupling in DNA polymerase information transfer

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SUMMARY

The extraordinary fidelity of DNA replication during forward polymerization and exonuclease error correction is largely a function of a conformational change that occurs in response to a correct dNTP binding to properly base-paired duplex DNA. The conformational change serves as a kinetic barrier to effect the rapid incorporation of correct bases while minimizing the rate of polymerization with incorrect bases and allowing for selective removal of mismatches. However, in spite of the number of attractive features to the conformational change model, the evidence in support of such a rate-limiting step is still subject to significant uncertainty. It is the challenge of further work on DNA polymerases as well as many other enzyme systems to devise new methods to define the transient state of the enzyme during catalysis and to relate the kinetic and thermodynamic parameters to the enzyme structure.

1. INTRODUCTION

Template-directed DNA polymerization occurs according to standard base-pairing rules but with a fidelity far greater than expected according to the free energy differences for correct and incorrect base pairs observed in solution (reviewed in Kirkwood 1986; Kornberg 1992). If the DNA polymerase simply zippered together those base pairs that formed in solution, the error frequency would average one mistake out of 200 bases polymerized. However, the observed fidelities of polymerization, defined as the inverse of the error frequency, are in the range of 10^5 to 10^6, and error correction by the proof-reading exonuclease can increase this to an overall fidelity of 10^8 to 10^9. The mechanistic basis for this extraordinary fidelity has been the subject of considerable speculation involving postulated reaction intermediates thought to be necessary to amplify the selectivity (Kirkwood 1986). However, until recently there has been no direct analysis of the polymerase reaction that would allow the identification of reaction intermediates or provide evidence to distinguish among the several postulated mechanisms.

Understanding the basis for DNA polymerase fidelity is actually a question of defining the kinetic, thermodynamic, and structural basis for selectivity. The problem can be posed in terms of establishing how the free energy differences in binding of the correct nucleotide versus an incorrect nucleotide can be translated into differences in rates of polymerization. Accordingly, the problem falls under the broader question of the utilization of binding energy for enzymatic specificity and catalytic efficiency.

In this report, we will summarize the kinetic and thermodynamic analysis of the reactions catalysed by T7 DNA polymerase. T7 DNA polymerase consists of a 1:1 complex between *E. coli* thioredoxin and T7 gene 5 protein, which contains both the polymerase and exonuclease active sites on a single polypeptide. Proof-reading by the exonuclease via selective removal of mismatched bases increases the overall fidelity of replication. As we shall see, selectivity during polymerization and exonuclease proof-reading is a function of a change in protein conformation in response to a correct base pair. This change in protein structure is dependent upon correct base pairing in the incoming triphosphate and in the template/primer duplex. The change in protein structure is thought to provide the mechanism by which the enzyme checks for proper Watson-Crick base-pairing geometry.

Studies on DNA polymerase I (Pol I) have indicated a mechanism with some similarities to the T7 pathway (Mizrahi *et al.* 1985; Patel 1991). However, there are also some significant differences which may reflect the primary role of Pol I as a repair enzyme rather than a polymerase involved in replication. For example, Pol I Klenow fragment shows essentially no discrimination in binding the correct versus the incorrect dNTP (deoxynucleoside triphosphate) (Kuchta *et al.* 1987) and the contribution of the exonuclease to fidelity is nearly negligible and occurs by dissociation and rebinding of the DNA rather than by intramolecular transfer (Joyce 1989). For these reasons, we will restrict our attention to the results on T7 DNA polymerase, which represents the most simple of the polymerases known to be sufficient for replication.

2. SINGLE TURNOVER KINETICS

To define the elementary steps leading to the incorporation of a deoxynucleoside triphosphate, it is

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necessary to examine the reaction on the timescale of a single enzyme turnover and with a sensitivity sufficient to detect events occurring at a single enzyme site. We have used rapid chemical quench-flow methods to examine the incorporation of a single base into synthetic oligonucleotides (Patel *et al.* 1991; Wong *et al.* 1991; Donlin *et al.* 1991). Most of these studies have employed the 25/36-mer shown in scheme I.

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GCCTCGCAGCCGTCCAACCAACTCA3′-OH
CGGAGCGTCGGCAGGTTGGTIGAGTAGGTCTTGTTT
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Scheme I. Synthetic oligonucleotide

3. STEPS IN THE POLYMERIZATION CYCLE

The elementary steps of the polymerization cycle are shown in figure 1 with the rate and equilibrium constants for the incorporation of dTTP into the 25/36-mer. Although the pathway adequately describes the reaction for any base pair, independent of DNA sequence, there are subtle differences in the reaction kinetics with different DNA sequences due to nearest neighbour interactions. For the present work, it was necessary to perform all of the studies using the same oligonucleotide to derive a single, self-consistent set of rate and equilibrium constants. Studies with other sequences have confirmed that the mechanism is universal and the variation in individual rate constants is small.

The binding of the correct dNTP to the E-DNA complex occurs by a two step process. The initial collision complex comes to a rapid equilibrium with a $K_d = 18$ μM for the correct dNTP. The binding of the correct dNTP then induces a change in protein conformation which is followed by a fast chemical reaction producing the elongated DNA and the by-product, pyrophosphate. After the chemical step, the enzyme returns to the ground state conformation and pyrophosphate is released. Translocation of the DNA to the site for the next incorporation is apparently too fast to observe, and therefore is not included as a distinct step in the pathway. Rather, we consider translocation as a free, rapid diffusion of the DNA between the two sites on the enzyme.

The pathway can be described in terms of two conformational states of the enzyme. In the ground state (E), the enzyme is free to rapidly diffuse between the sites for DNA synthesis and pyrophosphorolysis. The binding of dNTP or PP_i shifts the equilibrium binding of the DNA between the two sites by mass action. Thus, there is no need for a distinct, energy requiring translocation step. A different enzyme state (E*) is reached after binding the correct dNTP and can be thought of as the state where the enzyme locks down over the substrates and effects catalysis. Thus the key to fidelity is in the 'decision' of the enzyme to lock down over the incoming dNTP and DNA. In quantitative terms, most of the fidelity is a function of the conformational change, which requires proper base pairing in both the incoming dNTP and in the template or primer duplex DNA. Each of the steps contribution to the overall fidelity will be examined in more detail in the following sections.

4. SELECTIVITY OF NUCLEOTIDE BINDING

Recognition of the correctly base-paired dNTP occurs by a two step binding reaction. The first step is a rapid equilibrium involving the association of dNTP with the E–DNA complex. The dissociation constant for a correct base pair in the collision complex is approximately 18 μM, while that for an incorrect base pair is too high to measure accurately (4–8 mM). Thus, the

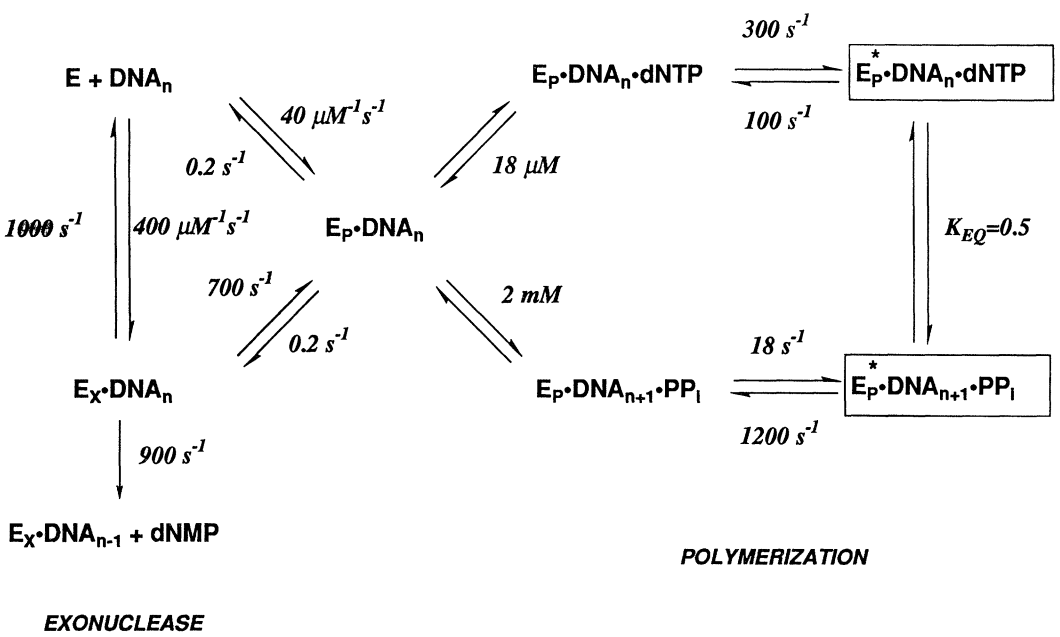

Figure 1. Pathway of DNA polymerization and exonuclease error correction. The pathway and rate constants are from Patel *et al.* (1991) as described in the text.

first binding step provides a selectivity of approximately 200- to 400-fold to the net fidelity of incorporation. It is interesting to note that the fidelity for this step is approximately the same as expected for the base pair formation in solution. The major difference is that the apparent free energy of base pair formation at the active site of the enzyme in the initial collision complex is identical for a G:C or an A:T base pair. Thus, it appears as though the enzyme somehow compensates for fewer hydrogen bonds formed in the A:T base pair.

5. CONFORMATIONAL CHANGE

The second step in the pathway limits the rate of incorporation at saturating nucleotide concentrations. The reaction occurs at a maximum rate of 300 s^{-1} for the correct dNTP, but at a rate of only 0.3 s^{-1} for a mismatch. Thus, the step that limits the chemical reaction contributes a factor of 1000 to the overall fidelity. It is possible that this step represents that actual chemical reaction; however, there are several lines of evidence leading to the conclusion that there is a change in protein conformation which is followed by a much faster chemical reaction. The existence of such a rate limiting conformational change has significant implications for the fidelity of replication because it allows the enzyme to increase the selectivity by making the conformational change dependent upon the proper base pairing geometry. Binding energy in the new conformational state is then used to effect catalysis. However, none of the evidence in support of a rate-limiting conformational change is unequivocal. In fact, it is exceedingly difficult to prove that such a step exists because of the very nature of the kinetics. If the chemical step is fast and follows a rate-limiting conformational change, then the two reactions cannot be resolved.

Our first piece of evidence in favour of a conformational change is based upon the observation of a change in protein fluorescence seen in the stopped-flow at a rate equal to the rate of incorporation of the dNTP (unpublished data). Because the change in tryptophan fluorescence probably reflects a change in the environment around one or more tryptophan residues, one is left with the suggestion that a change in conformation has probably occurred. However, this conclusion is subject to the argument that the fluorescence change could also be a function of the change in the local environment at the active site during the chemical reaction and not be an indicator of a change in protein structure. None the less, it is not unreasonable to suggest that a change in protein fluorescence always reflects a change in protein structure. In those cases where a fluorescence change has been correlated with chemistry, closer analysis may reveal a conformational change preceding the chemistry step. This appears to be the case for the myosin ATPase (Rosenfeld & Taylor 1984), and possibly for tryptophan synthase (Anderson *et al.* 1991).

The second piece of evidence suggesting a conformational change comes from the analysis of the kinetics of nucleotide binding by a millisecond pulse-chase experiments (Patel *et al.* 1991). During the pulse period, the E–DNA complex is reacted with radiolabelled dNTP. The reaction is then chased with an excess of unlabelled dNTP for a period sufficient to convert all of the tightly bound dNTP to products. The results of the pulse-chase experiment are then compared to a direct quenching experiment, which measures the amount of product formed at the time of the quench. The difference between the pulse-chase experiment and the direct quench experiment is a function of the amount of tightly bound dNTP. This experiment argues for the existence of an E*-DNA–dNTP state which is distinct from the collision complex in terms of the rate at which dNTP dissociates. That is, the observation of additional product formed during the chase period implies the existence of a dNTP species which dissociates more slowly than one estimates for the rate of dissociation from the collision complex (calculated from the minimum binding rate and the dissociation constant). Although this experiment is definitive in principle, it is difficult to perform and the magnitude of the signal is small.

The final piece of evidence is based upon analysis of the elemental effect due to a sulphur substitution in the dNTP. In principle, this method is based upon the assumption that the one can selectively slow down the rate of the chemical reaction without altering the rate of the conformational change or any of the parameters governing dNTP binding. The most commonly employed method in enzymology is based upon analysis of isotope effects, whereby substitution of a heavier isotope in the reactant may be expected to lead to a lower rate of reaction. For the reaction involved in DNA polymerization, none of the possible isotope effects are large enough to be examined by transient state kinetic methods; and the steady state methods are inappropriate because of the existence of several reactions which limit the rate in the steady state. Therefore, we have resorted to analysis of the elemental effect resulting from the substitution of sulphur for oxygen in one of the non-bridging oxygens of dTTP.

Our initial analysis of the sulphur elemental effect was based upon the suggestion that one should expect a 100-fold reduction in the rate of the reaction, based upon measurement of the rates of reaction in solution (Benkovic & Shray 1971). Thus, when a threefold sulphur elemental effect was observed, it was taken as evidence for a rate-limiting conformational change. That is, the rate of the chemical reaction was observed to be 70 s^{-1} with the dTTPαS, which implies that the rate of the chemical reaction with dTTP should have been 7000 s^{-1}. Therefore, the observed rate of 300 s^{-1} must have been due to a rate-limiting conformational change preceding chemistry (Patel *et al.* 1991). However, the initial analysis of the reaction rates of model systems in solution (Benkovic & Shray 1971) now appears to be in error; more complete analysis of model systems has indicated an elemental effect only three-to ten-fold for the rate of hydrolysis of a phosphate diester (Herschlag *et al.* 1991). Therefore, one could take our results to imply that the chemical

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reaction was totally rate limiting and there was no conformational change preceding.

More rigorous analysis of the expected sulphur elemental effect leads to a more subtle interpretation based upon an understanding of the transition state for phosphate transfer reactions. Phosphate transfer reactions in solution are thought to proceed by a pre-associative pathway via a metaphosphate intermediate (Herschlag & Jencks 1989, 1990). The magnitude of the sulphur elemental effect will be proportional to the extent of bond formation in the transition state. That is a more associative transition state would lead to a larger elemental effect, whereas a more dissociative transition state should lead to a lower elemental effect. One might expect the transition state at the active site of the enzyme to be markedly different from that observed in solution, due to the utilization of binding energy to carry out catalysis. Thus the magnitude of the elemental effect may be different at the active site of the enzyme than in solution.

It seems likely that the reaction at the enzyme site may proceed with a high degree of bond formation in the transition state which is a function of the utilization of substrate binding energy to effect catalysis. If a conformational change occurs, it may bring the reacting groups together forcing the reaction to proceed. In this case one may expect a large intrinsic elemental effect on the rate of the chemical reaction, but the magnitude of the effect will be obscured by the rate-limiting conformational change. On the other hand, the lack of a significant elemental effect may imply a larger extent of bond breakage in the transition state, which could be a function of the utilization of binding energy in the pyrophosphate binding pocket, whereby the binding energy between groups on the enzyme and the pyrophosphate moiety may facilitate bond cleavage. In this case one would observe the full elemental effect, but it would be small.

Because either explanation could account for the data, the small elemental effect result is apparently uninterpretable. However, further analysis of misincorporation and correct incorporation over a mismatch has produced considerably larger elemental effects (30- to 60-fold) (Wong *et al.* 1991). Thus, the larger elemental effect may imply that chemistry is largely rate limiting for misincorporation and these measurements may then provide the standard for the full extent of the intrinsic elemental effect at the active site of the enzyme. Complete analysis of the reactions involving the dNTPαS has shown that the binding constants are not changed and therefore the elemental effect appears to be on the rate of the chemical reaction and is not due to a steric effect of the sulphur substitution. Taken together, the results on correct incorporation and misincorporation are best interpreted in terms of a rate-limiting conformational change which dictates the rate of correct incorporation. With a mismatch, the incorrect dNTP fails to induce the change in protein conformation necessary to accelerate catalysis; and so, a misincorporation occurs at a slow rate reflecting residual catalytic potential due to the proximity of the bound dNTP.

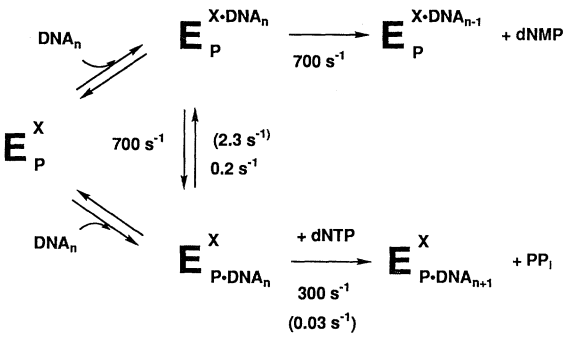

Figure 2. Kinetics of sliding between polymerase and exonuclease sites. The rates of movement of the DNA between enzyme sites for the polymerase (E_P) and exonuclease (E^X) are given. The rates in parenthesis are for the case of a mismatch at the 3′ end of the DNA primer.

Thus, the conformational change serves as a kinetic barrier to select the correct dNTP and provides a faster rate of incorporation relative to that of a mismatch. Although this interpretation is most satisfying, further more direct evidence for the conformational change is needed.

6. EXONUCLEASE ERROR CORRECTION

The exonuclease site is located on a separate domain approximately 25 Å† from the polymerase site (Ollis *et al.* 1985). For the DNA to transfer from the polymerase site to the exonuclease site, it must slide and melt out 6–8 base pairs of duplex. A comprehensive kinetic analysis of the reactions that govern exonuclease error correction has led to the scheme described in figure 2. The binding of the DNA to the polymerase site is thermodynamically preferred by approximately 3.5 kcal mol^{-1} ($K \simeq 300$). The transfer of the DNA from the polymerase site to the exonuclease site is normally a rare event (0.2 s^{-1}) relative to the rate of forward polymerization (300 s^{-1}). However, once in the exonuclease site, single stranded DNA is hydrolysed at a rate of 700–900 bases per second. The net rate is limited by the rate of movement of the DNA from the polymerase site.

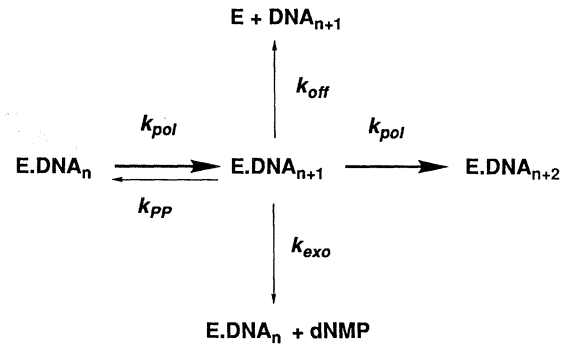

Figure 3. Kinetic partitioning or exonuclease error correction. The kinetic constants defining the possible fates of an E–DNA complex after each cycle of polymerization are shown. The rate constants are defined in the text.

† 1 Å = 10^{-10} m = 10^{-1} nm.

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Table 1. *Kinetic parameters for correct versus incorrect incorporation*

	correct[a]			incorrect		
	K_m/mM	k_{cat}/s^{-1}	[b]k_{obs}/s^{-1}	K^m/mM	k_{cat}/s^{-1}	[b]k_{obs}/s^{-1}
k_p	0.02	300	250	$\simeq$6–8	$\simeq$0.14	0.002
k	2	3	1	>20	—	<0.0001
k_p^{next}	0.02	300	250	0.08	0.025	0.012
k_{off}	—	—	0.2	—	—	0.4
[c]k_x	—	—	0.2	—	—	2.8

[a] All parameters for correct dNTP incorporation are from Patel *et al.* (1990). Excision rates are from Donlin *et al.* (1990).
[b] k_{obs} values for polymerization and pyrophosphorolysis are calculated at estimated physiological concentrations for dNTP of 100 μM and pyrophosphate of 1 mM, respectively.
[c] k_x is the observed rate of excision. The intrinsic rate of excision is very fast (700 s^{-1}). The observed rates cited here are limited by the rate of transfer of the DNA from the polymerase site to the exonuclease site.

Selectivity in error correction by the proof-reading exonuclease is dictated by the reactions occurring at the polymerase site (Donlin *et al.* 1991). The kinetic partitioning that defines the fate of a mismatched or correctly based paired DNA product is shown in figure 3, and the rate constants summarized in table I. At each step of polymerization, the E–DNA complex partitions among the four routes shown. The DNA can dissociate at a rate defined by k_{off}, another correct base pair can be added (k_{pol}), the reaction can reverse by pyrophosphorolysis (k_{PP}), or the DNA can slide to the exonuclease site (k_{exo}). Following the formation of a mismatch, the rate of correct polymerization on top of the mismatch is reduced to approximately 0.01 s^{-1}, and the rate of movement into the exonuclease appears to be slightly accelerated (2.8 s^{-1}). Analysis of this kinetic partitioning shows that the exonuclease contributes an additional factor of 200 to the overall fidelity of replication, calculated as the probability of excising a mismatch relative to the rate of polymerization on top of the mismatch. It is remarkable that this increase in fidelity is achieved at very little cost, calculated as the probability of removing a correctly paired base relative to the rate of polymerizing on top of it (0.2 to 300). This selectivity is largely attributable to a 20 000-fold reduction in the rate of correct incorporation when there is a mismatch in the template or primer. Thus, if the fidelity is a function of the rate-limiting conformational change, then these results imply that the conformational change is sensitive to disruption of the Watson–Crick helix in the template.

The exonucleases is not processive. That is, it removes only one base from the 3′ end of the DNA during each visit to the exonuclease site. Thus, although the rate of movement of the DNA into the exonuclease site limits the rate of hydrolysis, the DNA does not stay in the exonuclease site long enough to remove more than one base because the rate of return to the polymerase site is approximately equal to the rate of the exonuclease. Even given these kinetic constants, one would expect to see a series of products in geometrically decreasing intensities resulting from sequential exonuclease reactions. That is, if 50% of the DNA is cleaved in one visit to the exonuclease site, then 50% of the products of the first cleavage reaction would be cleaved again. The fact that we cannot detect products of multiple reactions in a single turnover experiments may suggest additional features of the exonuclease that limit the processivity of the reaction. For example, it is known that dNMP (the product of the exonuclease) binds at the exonuclease site with a moderate affinity. Therefore, it seems likely that there may be an ordered release of the DNA and then the dNMP from the exonuclease site following hydrolysis. The presence of the dNMP would effectively prevent the second hydrolysis reaction.

Overall, the exonuclease achieves its selectivity by virtue of the reactions occurring at the polymerase site. By our model, the exonuclease is an essentially dumb enzyme site that shows no selectivity in hydrolysis. Indeed, if the duplex DNA must be melted out to get it into the exonuclease site, any information pertaining to correct base pairing must be lost. All of our analysis points to the conformational change at serving as a gate to control the editing process. A mismatch in the 3′-terminal base-pair prevents the enzyme from closing down over the DNA to effect catalysis. The polymerization reaction effectively stalls, allowing time for the DNA to be transferred into the exonuclease site for error correction. More recent information has indicated that mismatches as far as three base pairs from the 3′-end still inhibit the polymerization reaction and contribute to the net fidelity (unpublished observations). These results suggest that the conformational change may be rather global in that it involves a large segment of protein overlapping at least four base-pairs of the DNA at the active site. Mismatches anywhere in this segment inhibit or prevent the change in enzyme structure necessary to effect catalysis.

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Discussion

R. N. F. Thorneley (*AFRC Division of Nitrogen Fixation, University of Sussex, Brighton, U.K.*). I remember a discussion in Gottingen about 20 years ago, about apparent second-order rate constants of proteins binding to DNA, and there was a stocastic argument that apparent rate constants were being found faster than diffusion limitation. There should be, I think, a square root dependence on the length of the DNA and I wondered if Professor Johnson sees any dependence for his protein–DNA binding rate constants on the length of the DNA?

K. A. Johnson. The one constant that is approaching the diffusion limit is the rate of binding into the exonuclease site, $\approx 10^8\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$. We have not looked at that as a function of DNA length.

R. N. F. Thorneley. I think the point Eigen was making at Gottingen, and this may be well characterized now, was that the protein can have an interaction on the DNA well away from the final binding site. Then the protein creeps along the DNA. Professor Gutfreund knows more about this.

H. Gutfreund. Diffusion limits are a bit like problems in physical organic chemistry. If you know the answer, you can explain it. Many theories have been written in the last 10 years about how proteins can walk along DNA, but the diffusion limit depends on the diffusion constant of the smallest component, and for a protein binding, $10^8\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ would be quite a reasonable diffusion rate constant, even without any electrostatic interactions.

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