Stabilization of T4 polynucleotide kinase by macromolecular crowding

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ABSTRACT
      T4 polynucleotide kinase rapidly loses activity during its reaction on
duplex DNA termini. Addition of high concentrations of nonspecific polymers
reverses or prevents this inactivation. In contrast, additions of related
materials of lower molecular weight are relatively ineffective in stabilizing
the kinase. Such a pattern suggests that the stabilizing effects of polymers
on kinase activity are due to macromolecular crowding. An effect of crowding
on the known tendency of the kinase to undergo oligomerization reactions is
consistent with our observations.

INTRODUCTION

      The enormous concentrations of macromolecules within cells can cause

changes in equilibria or rates of reactions (1). As part of a series of

experiments to assess the importance of these effects in systems involving

nucleic acids, we have examined the in vitro effects on T4 polynucleotide

kinase (E.C. 2.7.1.78) of high concentrations of several nonspecific macro-

molecules. Such additions cause a striking increase in kinase activity which

is shown to result from a stabilization of the enzyme under conditions where

it has a high turnover number.

MATERIALS AND METHODS

Materials

      Restriction nucleases were purchased from Bethesda Research Laboratories.

Ficoll 70, ADP, ATP, T4 polynucleotide kinase and T4 DNA ligase were from

Pharmacia; E. coli alkaline phosphatase (BAPC), and pancreatic RNase A from

Millipore; PEG 200 (nominal molecular weight 190-210), PEG 8000 (nominal

molecular weight 7000-9000), and imidazole from Baker; [γ-^{32}P] ATP from New

England Nuclear; and dithiothreitol from Calbiochem. pBR322 DNA was either a

gift from Dr. M. Gellert or the product of Bethesda Research Laboratories or

Pharmacia. The commercial preparations contained small amounts of relatively

low molecular weight materials which were highly labeled by T4 PNK. These

materials were routinely removed by treatment with boiled pancreatic RNase A
(2) and the DNA reisolated.

Methods

Assay of the kinase exchange reaction. Unless otherwise specified, the
exchange reaction of T4 PNK was assayed at 37° for 20 min in polypropylene
tubes (Eppendorf) in mixtures (final volume of 10 µl) containing 50 mM
imidazole-HCl buffer (pH 6.4), 4.5 mM dithiothreitol, 18 mM $MgCl_2$, 0.1 mM ADP,
12 µM [γ-^{32}P] ATP (2-20 Ci/mmol), 0.1 µg of the specified DNA, polymers as
indicated, and 0.1 volume of kinase dilution in a diluent (3) of 50 mM Tris·HCl
(pH 7.6) - 10 mM β-mercaptoethanol - 0.5 mg/ml of bovine plasma albumin.
(Reaction mixture composition is a minor modification of that of Berkner and
Folk (4,5) except for the addition of polymers.) Kinase dilutions in the
above diluent were stable for at least 20 min on ice. The reaction mixtures
were assayed for radioactivity migrating at the position of the specified DNA
on agarose gels or for acid-precipitable radioactivity, or for both. Auto-
radiograms were quantitated with a Joyce-Loebl recording microdensitometer;
lanes with serial dilutions of labeled DNA standards were present on each gel
for calibration.

RESULTS

High Concentrations of Polymers Increase the Exchange Reaction of T4 Poly-
nucleotide Kinase on DNA

Addition of high concentrations of any of several polymers caused large
increases in the amount of exchange labeling of DNA termini by T4 PNK. PEG
8000, rabbit liver glycogen, or Ficoll 70 were all active, whereas similar
concentrations of related materials of lower molecular weight had little
effect (Fig. 1). Such a pattern is consistent with macromolecular crowding
as a basis for the effect of polymers on the kinase (see Discussion). The
increases in the exchange reaction due to the polymers can be very large. For
example, it requires 100-1000 times more kinase reacting in the absence of PEG
to make the amount of product formed by the kinase in the presence of 6% w/v
PEG 8000 (20 min incubations with recessed 5'-termini formed by Pst I nuclease
on pBR322 DNA).

The effect of polymers is quite general for kinase reactions on higher
molecular weight DNA. Increases of exchange labeling in the range of 10- to
1000-fold are readily obtained in PEG solutions on recessed, protruding or
blunt-ended 5'-termini of duplex DNA, or on 5'-termini at single-strand breaks
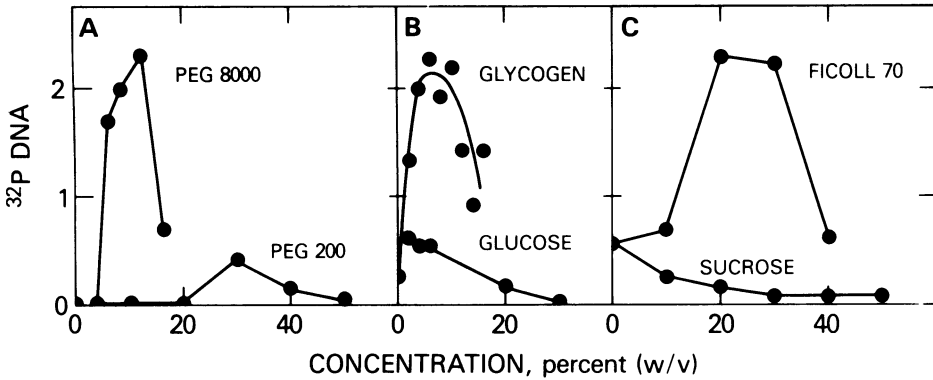(i.e. at "nicks") in duplex DNA. Both the forward and reverse kinase reactions

Figure 1. Comparison of effects of high molecular weight polymers with those of low molecular weight derivatives in exchange-labeling by T4 polynucleotide kinase of recessed DNA termini. Kinase exchange assay mixtures as in Methods contained Pst I nuclease-digested pBR322 and the additions indicated. Kinase concentrations were 170 units/ml in B and C and 1.7-170 units/ml in A. The values on the ordinates are expressed on arbitrary linear scales; those values in A are are normalized for the amounts of kinase present.

are stimulated as described below. The polymer effect diminishes with shorter DNA molecules: reaction of DNA substrates of < 150-300 base pairs is little affected by the presence of PEG 8000.

Increased Kinase Activity is Due to Stabilization of the Kinase by Polymers

The apparent stimulation of kinase activity is due to a stabilization of the kinase by the polymers at incubation temperatures where the kinase has a high turnover number on duplex DNA substrates. For example, at lower tempera-tures (0 or 20°) the rate of the exchange reaction is low but that rate is essentially constant for periods of hours--and is little affected by the presence of PEG. In contrast, at 37° the rate increases tremendously but the enzyme becomes inactive within minutes unless a polymer like PEG 8000 is present (Fig. 2). High stable rates were also obtained at 45° in 6% w/v PEG 8000 but not at 55°. A second addition of DNA is without effect in complete reaction mixtures in which the kinase has been inactivated by preincubation for 10 min at 37° without PEG (cf. ref. 4).

PEG can also reactivate enzyme which has become inactive due to prior incubation at 37° in reaction mixtures without PEG. Within a few minutes after addition of PEG to a reaction mixture which had been preincubated 10 min at 37° to inactivate the kinase, the rate of the exchange reaction increased > 30-fold (Fig. 3) to at least a third of that of PNK assayed directly in PEG solutions.
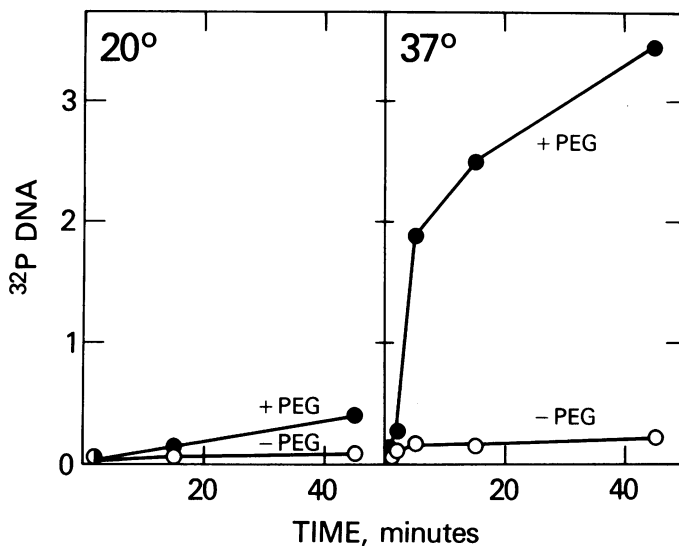
Figure 2. Effect of PEG 8000 at 20° vs. 37° on exchange-labeling of recessed DNA termini. Kinase exchange assay mixtures as in Methods contained Pst I nuclease-digested pBR322 DNA and 170 units/ml of kinase. Where indicated, 6% w/v PEG 8000 was present. After incubation at the indicated temperatures, reaction mixtures were assayed by the gel procedure. The ordinate is an arbitrary linear scale proportional to the radioactivity incorporated into linear pBR322 DNA. The decreased rate at longer times with PEG at 37° is due to extensive reaction of the substrate and was not seen in experiments with lower enzyme levels. Incorporation of ^{32}P in assays at 0° were > 100-fold lower with or without PEG.

Polymers Do Not Change the Nature of the Kinase Products

The ^{32}P-labeled DNA products made by the kinase in the presence of 6% w/v PEG 8000 were characterized as follows: Either a Sph I or Pst I nuclease digest of pBR322 DNA was labeled by the PEG-stimulated kinase exchange reaction and the kinase products were ligated by T4 DNA ligase. The substrate was extensively ligated as shown by its altered mobility on agarose gels. The ligated products were then recut with the same restriction nuclease that had been used to form the original 5'-termini. The result was that the ligated products were completely recut. When the recut sample was further tested, it was found to be sensitive to alkaline phosphatase. These results indicate that the structure of the termini is not altered by the labeling procedure, that the labeled termini are substrates for DNA ligase, and that the label is exactly at the expected point of restriction, and not, for example, at adventitious nicks or at exonuclease-trimmed ends.
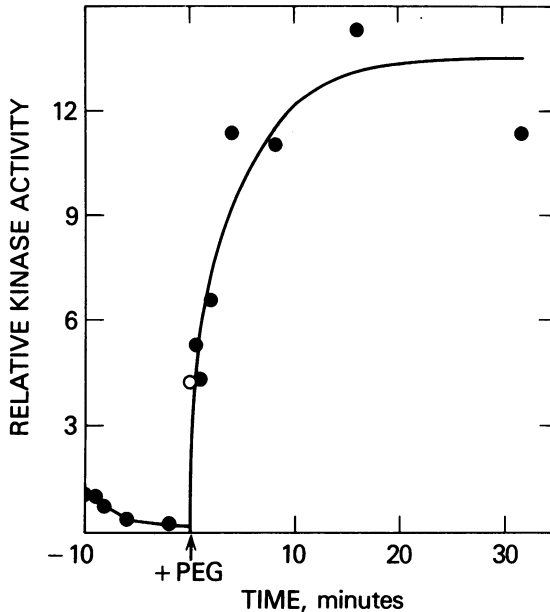
Figure 3. Kinetics of inactivation of T4 polynucleotide kinase in the absence of PEG and its reactivation upon addition of 6% w/v PEG 8000. A large-scale incubation mixture (containing Pst I nuclease-digested pBR322 DNA and 170 units/ml of T4 polynucleotide kinase) which was identical to the kinase exchange mixture in Methods except that the ATP was unlabeled, was incubated at 37° in the absence of PEG. At the arrow, 22.5 µl of 18% w/v PEG 8000, 50 mM imidazole buffer (pH 6.4), 4.5 mM DTT, 18 mM MgCl$_2$, 0.1 mM ADP and 10 µg/ml Pst I nuclease-digested pBR322 was added to 45 µl of this incubation mixture. At the indicated times before and after this addition, aliquots of 5 µl or 7.5 µl, respectively, were removed for exchange assays with Pst I nuclease-digested DNA just as in Methods (in 10 µl volumes) except that a 1 min incubation at 37° was used. Samples that did not contain PEG were assayed in the absence of PEG, whereas those taken from the incubation mixture after addition of PEG were assayed in the presence of 6% w/v PEG 8000. The kinase concentration during assay of either set of samples was 85 u/ml. Activities are expressed on an arbitrary linear scale. The open symbol is the activity in a 1 min assay in 6% w/v PEG 8000 of a kinase sample that had not been preincubated at 37°.

Estimation of the Equilibrium Constant of the Kinase Reaction in 6% w/v

PEG 8000

The augmented forward and reverse reactions in PEG-containing solutions allow a ready approach to equilibrium from both sides of the reaction. Concentrations at equilibrium correspond to $K = \frac{(P-DNA)(ADP)}{(HO-DNA)(ATP)} \cong 60$. This estimate for the equilibrium constant in PEG solutions on protruding 5'-termini of

high molecular weight DNA is similar to values reported for the reaction cata-
lyzed by T4 PNK in the absence of PEG with a small oligomer (K ≅ 50) (6) or
with a short segment of duplex DNA with protruding 5'-termini (K ≅ 33-37) (7).
Forward, Reverse, and Exchange Reactions of the Kinase are Affected Similarly
by PEG 8000

The relative lack of effect of crowding on the equilibrium constant
suggests that the forward and reverse reactions might be increased by similar
factors by PEG. We have assayed those rates individually and find two corre-
lations. First, in the absence of PEG, both the forward and reverse reactions
on Pst I nuclease-digested pBR322 DNA stop within a few minutes at 37° (as also
shown above for the exchange reaction). Second, in the presence of 6% w/v
PEG 8000, all three reactions proceed at similar high rates of activity.
Hence, the PEG effect on the exchange reaction is accompanied by generally
similar effects on both of the component reactions.

DISCUSSION

Certain duplex DNA substrates such as nicked DNA or DNA with recessed
5'-termini are refractory to kinase action. The rate of kinase action on
such substrates increases rapidly with increased temperature, but the enzyme
becomes unstable. The major effect of PEG and other polymers seems to be to
stabilize the kinase under conditions where it has a high rate of reaction.

The effects of PEG and the other polymers on the T4 PNK reaction seem to
be primarily due to their volume exclusion properties. Concentrated solutions
of three polymers of very different chemical properties, PEG 8000, glycogen,
and Ficoll 70, have large effects on T4 PNK, whereas low molecular weight
derivatives are relatively ineffective. This effect of high concentrations of
nonspecific high molecular weight polymers is consistent with macromolecular
crowding (1) as the basis for the kinase stimulation and this will be assumed
in the following discussion. However, we note that a fourth high molecular
weight polymer, bovine plasma albumin, did not have significant effect on the
T4 PNK reaction.

How might crowding stabilize the kinase? For two reasons, we suggest that
at least part of the stabilization due to crowding is a result of increased
binding between the kinase and DNA. First, previous studies (8,9) have shown
that the heat-stability of T4 PNK in non-crowded solutions is increased in
the presence of DNA. Second the magnitude of our stabilizing effect is
dependent on the specific type of DNA terminus that is present, again implying
that the binding of the enzyme to DNA is involved in the stabilization. There

are a number of ways in which the binding between kinase and DNA could be increased. Crowding might increase the effective concentrations of both enzyme and DNA or might change the conformation of the kinase. Alternatively, crowding could increase DNA binding by favoring self-association of the kinase—if an aggregated form of the enzyme bound more firmly to the DNA than did the dissociated form of the enzyme. Such a mechanism based on increased aggregation of the enzyme is consistent with several observations in the literature and has some support in our experiments. First, Minton and his collaborators have demonstrated shifts in the self-association equilibria of several enzymes or other proteins due to crowding (see ref. 1 for a review). They have also found stabilizing effects of crowding in several systems and discuss potential mechanisms (10). In addition, T4 PNK was shown by Lillehaug (11) to undergo aggregation reactions even in non-crowded solutions in the absence of DNA; conditions which favored self-association from monomer to tetramer were previously shown to cause increased kinase activity (12). Finally, the following observation supports our suggestion that crowding acts by shifting the aggregation equilibrium of the kinase. We find that at very high kinase concentrations (850 or 1700 T4 PNK units/ml)—where self-aggregation of the enzyme should be favored in noncrowded solutions—the PEG effect disappears, i.e., product formation at 37° is proportional to time and not changed by addition of 6% w/v PEG 8000.

There are obvious preparative advantages to using polymers in labeling or dephosphorylation of DNA. A description of these techniques is in preparation.

ACKNOWLEDGEMENTS

ABBREVIATIONS

PEG, polyethylene glycol; bp, base pair; T4 PNK, T4 polynucleotide kinase.

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