Glutamate Overcomes the Salt Inhibition of DNA Polymerase III Holoenzyme*

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Even though Escherichia coli can grow in media containing up to 1 M NaCl, one-fifth that amount of NaCl will completely inhibit the in vitro activity of DNA polymerase III holoenzyme. It has been established that the major intracellular ionic osmolytes are potassium and glutamate (Richey, B., Cayley, D. S., Mossing, M. C., Kolkka, C., Anderson, C. F., Farrar, T. C., and Record, M. T., Jr. (1987) J. Biol. Chem. 262, 7157–7164). We have found that holoenzyme catalyzes replication efficiently in vitro in up to 1 M potassium glutamate. Two salt effects on the replication of single-stranded DNA were observed. At low salt replicative activity was enhanced and at high salt there was anion-specific inhibition. We have found that DNA polymerase III holoenzyme tolerated 10-fold higher concentrations of glutamate than chloride. The ability of various anions to extend the useful range of salt concentrations followed the order: phosphate < chloride < N-Ac-glutamate < acetate < glycine < aspartate < glutamate. With the exception of phosphate, this order followed the Hofmeister series indicating that the anion-specific effects were due to anions interacting at the protein-water interface at weak anion binding sites. Glutamate did not reverse the inhibition by chloride. The low salt enhancement and high salt inhibition effects were additive for the two anions indicating that they competed for common anion binding sites. The major salt-sensitive step was holoenzyme binding to template rather than the subsequent elongation reaction.

The most complex of the DNA polymerases in Escherichia coli, the multisubunit DNA polymerase III holoenzyme, is responsible for the majority of replicative DNA synthesis as established by biochemical and genetic studies (for reviews, see Kornberg, 1980, 1982; McHenry, 1985; McMaken et al., 1987; McHenry, 1988a). The precise subunit structure is still not fully characterized although holoenzyme1 is probably a dimer consisting of 7 to 10 different subunits (McHenry, 1982; Hawker and McHenry, 1987; McHenry, 1988a, 1988b; Maki and Kornberg, 1988a) with distinct leading and lagging strand halves. Although there are only about 20 copies/cell (McHenry and Kornberg, 1977), the ability of holoenzyme to incorporate at least 150,000 nucleotides before dissociating from a template (Mok and Marians, 1987) more than compensates for its low cellular abundance. As yet unreconciled is the extreme salt sensitivity of holoenzyme. Even though E. coli can grow in media containing up to 1 M NaCl (Epstein and Schultz, 1965; Munro et al., 1972; Richey et al., 1987), potassium or sodium chloride at concentrations as low as 200 mM can completely inhibit holoenzyme activity in vitro (Burgers and Kornberg, 1982; Crute et al., 1983; Kwon-Shin et al., 1987).

The molecular basis for the in vitro salt sensitivity of holoenzyme activity has not been established even though this sensitivity has been used as a tool in several studies for resolving the steps involved in DNA replication. ATP (Burgers and Kornberg, 1982; Crute et al., 1983), SSB (Burgers and Kornberg, 1982), and the β subunit (Crute et al., 1983) allow holoenzyme activity to tolerate somewhat higher salt concentrations. Since these three effectors also lead to a greater stabilization of initiation complex formation, one might erroneously conclude that after initiation complex has been formed holoenzyme is less sensitive to salt than prior to complex formation. We present evidence here that holoenzyme-SSB association is intrinsically salt dependent in a manner similar to other protein-DNA interactions. Our investigation of the molecular basis of the holoenzyme salt sensitivity is prompted by recent work showing that potassium and glutamate are the major free ions in E. coli and are the intracellular osmolytes that increase in response to extracellular osmotic pressure (Record et al., 1985). We tested whether glutamate was less inhibitory to holoenzyme activity than chloride. The relative effects of glutamate and chloride have already been compared in several protein-DNA interactions. The range of potassium concentrations over which 10 restriction enzymes are effective is broadened by the substitution of glutamate for chloride (Leirmo et al., 1987). Analysis of E. coli RNA polymerase activity in a kinetic assay indicates that the salt-sensitive step is the initial binding event that leads to closed promoter complex formation. Subsequent steps involving rearrangement to the open promoter complex are not particularly salt sensitive (Leirmo et al., 1987). A spectroscopic examination of SSB binding to ssDNA indicates that the intrinsic association constant is salt sensitive while the cooperativity parameter is not nearly as sensitive (Overman et al., 1988). The salt-sensitive step is the protein-DNA binding interaction and not cooperativity which primarily involves protein-protein interactions. Both of the above studies note marked differences in the ability of various anions to lower the protein-DNA binding constants. When the anions are ranked by their ability to lower the binding constant, the order follows the Hofmeister series: hypochlor-

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1 The abbreviations used are: holoenzyme, E. coli DNA polymerase III holoenzyme; SSB, E. coli single-stranded DNA-binding protein; ssDNA, single-stranded DNA; Ac, acetyl; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol.
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ide > iodide > nitrate ≈ bromide > chloride > acetate >
fluoride > glutamate. Phosphate and sulfate were not tested
in the protein-DNA binding interactions mentioned above
but comparison with the original Hofmeister series (Hofmei-
ser, 1888) predicts that they would have an effect equivalent
to glutamate in the above ranking.

The Hofmeister series is an empirical ranking of ions which
is related to their ability to interact with the first layer of
water at a protein surface (von Hippel and Schleich, 1969;
Record et al., 1978; Collins and Washabaugh, 1985). For a
protein to respond to solutes in this manner suggests the
importance of the rearrangement of water molecules and ions
during the interaction being examined. In this report we show
that glutamate and chloride bind to the same anion binding
sites on holoenzyme in a manner consistent with this being a
Hofmeister effect. We also demonstrate that the amino moieties
glutamate is an important factor in lowering the affinity of
glutamate for holoenzyme.

**Experimental Procedures**

**Materials**—Unlabeled and labeled nucleotides were obtained re-
spectively from Pharmacia LKB Biotechnology Inc. and ICN Ra-
diochemicals. Bio-Gel A-5m was from Bio-Rad, hydroxylapatite was from
Clarkson Chemical Co., and NuSieve-agarose was from FMC BioProducts.
All salts were purchased from Sigma and were made up in the fol-
lowing stock concentrations (all were adjusted to pH 7.5 ±
0.2 with 10 mM KOH, if necessary): 5 mM potassium glutamate, 4 mM
potassium acetate, 4 mM potassium aspartate, 2 mM glycine, 3 mM N-Ac-
glutamate, 2 mM potassium chloride and 1 mM potassium phosphate.

Enzyme dilution buffer was composed of 50 mM HEPES (adjusted to
pH 7.5 with 10 mM KOH), 20% (v/v) glycerol, 10 mM DTT, 200 µM/ml
bovine serum albumin, and 0.02% (v/v) Nonidet P-40. Conductome-
try of enzyme dilution buffer indicated that it had the equivalent of
35 mM KCl.

**Proteins and the DNA Template—Holoenzyme was isolated by the
method of Oberfelder and McHenry (1987). Two preparations were
utilized in this study and only minor differences were noted between
them. The following were isolated by standard procedures: anti-β IG
(Johanson and McHenry, 1980), DNA polymerase III (110,000 units/mg;
McHenry and Crow, 1979), and M13Gori ssDNA (Johanson and
McHenry, 1984). M13Gori ssDNA was a chimera DNA which consists of
a 6407-nucleotide M13 phage into which a 2216-nucleotide frag-
ment containing the G4 dnaG-dependent origin for complementary
strand DNA synthesis was inserted (Kaguni and Ray, 1979). It was
this dnaG dependence that formed the basis for the standard holo-
enzyme assay.

SSB was isolated from an overproducer, RLM727, by a modifica-
tion of the method of Meyer et al. (1980). The blue dextran-Sepharose
column was followed by a hydroxylapatite column (1 ml of column
material/5 mg of protein) to remove any blue dextran that may have bled
from the previous column. The column was equilibrated in 50
mM imidazole (pH 6.8), 20% glycerol, 1 mM NaCl, 5 mM β-mercapto-
ethanol, the sample was loaded, and the protein was washed with a
one-column volume of equilibration buffer. The protein was eluted with
eight-column volumes of equilibration buffer plus 70 mM potassium
phosphate. The isolated protein was then dialyzed against 50 mM
Tris (pH 7.5), 20% (v/v) glycerol, 100 mM NaCl, 1 mM EDTA, 1 mM
DTT.

**Primase (95% homogenous; 560,000 units/mg)** was isolated from
an overproducer RLM757 (Wold and McCallen, 1982), by a modi-
fication of the method of Rowen and Kornberg (1978). In the modified
procedure the sequence of chromatography was phosphocellose,
Bio-Rex 70 and DEAE-Sephalac, thus the phosphocellose column
replaced the DNA cellulose and valyl-Sephacel columns. The phos-
phocellose column (1 ml of column material/5.5 mg of protein) was
equilibrated with 50 mM imidazole (pH 6.8), 20% glycerol, 1 mM
EDTA, 1 mM DTT, and 25 mM NaCl. Sample was loaded and eluted with a
eight-column volume gradient from equili-
bration buffer to 50 mM imidazole (pH 6.8), 20% glycerol, 1 mM
EDTA, 1 mM DTT, and 300 mM NaCl. 0.5 column volumes/ll
Primase activity eluted at 220 mM NaCl.

**Holoenzyme Assay—** One unit of holoenzyme (or primase) was
defined as the amount needed to incorporate 1 pmol of (total) nucleo-
tide/min into acid-precipitable DNA under conditions where all other
components were saturating. In the assay priming occurs concomi-
tantly with holoenzyme replication and the amount of DNA replicated
was allowed to accumulate for 5 min at 30°C (Johanson and Mc-
Henry, 1980; Bouché et al., 1975). The final reaction contained: 50
mM HEPES, 16 mM Tris (pH 7.5), 12 mM NaCl, 15% (v/v) glycerol,
6 mM MgCl2, 0.012% (v/v) Nonidet P-40, 120 µg/ml valyl-Sephacel
albumin; 0.2 mM EDTA, 10 mM MgCl2, 542 pmol of nucleotide
ssDNA, 3.7 µM SSB monomers, 48 µM dATP, dCTP, and dGTP, 18
µM dTTP, [3H]dTTP added to a specific activity of 520 cpm/pmol
dTTP, 200 µM dNTPs, 40 units of primase, and limiting amounts
(<70 units) of holoenzyme. The salt in the assay was from storage
buffer of some of the components of MA 20-pH DNA of all components
except holoenzyme, kept on ice, were added to each tube containing
5 µl of various amounts of holoenzyme or salt. After 5 min at 30°C
the reaction was terminated and the DNA collected and quantitated
as previously described (Johanson and McHenry, 1980). To measure
the kinetics of the replication reaction a premix of all components
2.5% NuSieve-agarose, excess from the gel, the gel was melted,
was added to the premix to initiate the reaction. At various time inter-
vals, 25 µl was removed and the reaction quenched by adding it to 2 drops
of 0.2 M NaPF6, and putting it on ice.

In the holoenzyme assay 82% of the replicated DNA was retained
on the filters and all values have been adjusted accordingly. The
inhibition of the filters was examined by adding [3H]
M13Gori double-stranded DNA that had been separated from unin-
corporated [3H]dTTPs on a Bio-Gel A-5m (1.1 × 13 cm) gel filtration
column. The void volume fractions were pooled and either doted onto
dry GF/C filters or added to 25 µl of enzyme dilution buffer and
filtered by the standard procedure.

**Elongation Reaction Kinetics—** Two types of elongation complexes
were formed. In the two-step reaction, the initiation complex was
formed for 5 min at 30°C in a premix of all components excluding
dNTPs and including 500 µM ATP. In the three-step reaction,
the first step was synthesis of primed template for 5 min at 30°C and
the second step was initiation complex formation following the ad-
dition of holoenzyme and 500 µM ATP for 5 min at 30°C. The
initiation complexes were diluted into prewarmed tubes. A pre-
warmed mixture of anti-β IgL, dNTPs, and salt was added to initiate
the reaction. Anti-β IgL prevents the cycling of holoenzyme from a
decomposed duplex circle to a nonreplicated, primed circle (Johanson
and McHenry, 1980). In a control with anti-β IgG added to the
premix, it prevented elongation of 95% of the circles. The following
differed from conditions in the holoenzyme assay: 7.5 mM MgAC
500 µM ATP, 115 units holoenzyme, and 340 µM/ml anti-β IgG.

To test whether the rapid burst of DNA synthesis following the
addition of dNTPs to initiation complex was indeed due to
synchronous DNA replication, the two-step elongation reaction was
examined in a holoenzyme processivity experiment (as per Fig 3 of Fay et
al., 1983). A similar experiment was carried out at a
unique site, the distance traveled from the origin was determined by
restriction enzyme analysis. Replication was allowed to proceed for
5, 10, 15, 20, 25, 60, and 120 s. Subsequent Sphi digestion generated
restriction fragments in the following order: 592, 190, 2963, 2161, 961,
and 2335 base pairs. The fragments were electrophoretically separated
on 3% acrylamide gels, stained with ethidium bromide, the acid-precipitable DNA was collected on filters as in the holo-
enzyme assay, and the incorporated radioactivity determined. Adding
120 mM chloride decreased the first and last fragments generated to
44 and 42%, respectively, of the moles generated in the absence of
salt. Even though an inhibitory amount of chloride reduced the total
amount of DNA molecules replicated, the process-
ating holoenzyme molecules was not affected since the first and last
fragments were reduced to the same extent. The limitation of
the assay was that we could not make conclusions about the salt effect
during replication of the first ~1500 nucleotides since this was the
distance that needed to be replicated before the 592-base pair frag-
ments could be generated by Sphi restriction.

**DNA Polymerase III Assay—** The final 25 µl reaction mixture
contained 35 mM HEPES (pH 7.5), 15 mM MgCl2, 15% (v/v) gly-
cerol, 7 mM DTT, 70 µg/ml bovine serum albumin, 0.014% (v/v)
Nonidet P-40, 6.4 mM KC1, 80 µM dATP, dCTP, and dGTP, 30 µM dTTP,
[3H]dTTP added to a specific activity of 520 cpm/pmol dTTP, 188
µM/ml ascorbic acid, 50 mM NaCl, 200 µM dNTPs, and
add salt at the indicated concentrations. After 5 min at 30°C
the reaction was terminated and assayed as described in the holo-
enzyme assay. One unit of DNA polymerase III was defined as
the amount needed to incorporate 1 pmol of (total) nucleotide/min on an
activated salmon sperm DNA template (McHenry and Crow, 1979).
The activity of enzyme in the above-described buffer system was 90% of that in the usual buffer system of McHenry and Crow (1979).

RESULTS

Salt Titration of Holoenzyme Reaction Kinetics—Recent studies had shown that several protein-DNA interactions were less salt-sensitive to glutamate than chloride (Leirmo et al., 1987; Overman et al., 1988) and that glutamate is the major intracellular anion in E. coli while chloride is maintained at very low levels (Richey et al., 1987). Guided by these studies, we tested whether holoenzyme was likewise less sensitive to glutamate. To more fully characterize the effect of chloride and glutamate on holoenzyme activity, the time course of the reaction was examined. In the absence of added salt the kinetics of the standard reaction were sigmoidal (Fig. 1, A and B). The lag in the kinetics could be due to either the slow action of primase or the slow formation of initiation complex or a combination of both. Addition of low concentrations of salt resulted in higher percents of replicated DNA at all time points but did not remove the initial lag from the time dependence. Salt concentrations in excess of 50 mM chloride or 250 mM glutamate resulted in a diminution of replicated DNA at all time points (Fig. 1, A and B) such that 1 M glutamate reduced the rate to the same extent as 100 mM chloride. Two salt effects on holoenzyme activity were seen (Fig. 1C): enhancement at low salt and inhibition at higher salt. With glutamate as the anion the range over which holoenzyme remained active was greatly extended when compared to chloride. Since the high salt inhibition occurred at lower chloride concentrations, the maximum percent enhancement due to chloride was also less when compared to glutamate.

A variety of salts were tested in the holoenzyme assay and the ability of the anions to cause inhibition followed the order: phosphate > chloride > N-Ac-glutamate > acetate > glycine > aspartate > glutamate. The concentrations of these salts at which maximum activity was reduced by 50% were, respectively: 60, 110, 145, 250, 450, 720 mM, and 1 M Chloride, acetate, and glutamate followed the same anion ranking established in studies of other protein-DNA interactions (Leirmo et al., 1987; Overman et al., 1988). Even though a comparison with the classical Hofmeister series would rank phosphate as nearly equivalent to glutamate, we found that phosphate was more inhibitory than chloride. Phosphate inhibition of holoenzyme probably occurs by a mechanism different from the other anions.

Glutamate at Low Concentration Increased the Activity of Holoenzyme—To study further the activity enhancement by low salt, we performed a titration of holoenzyme in the presence or absence of 100 mM glutamate. In the absence of added salt the titration curve was sigmoidal (Fig. 2) due to the nonlinear time course of the reaction as seen in Fig. 1 and not due to cooperativity. From 15 units of holoenzyme to saturation at 60 units, 100 mM glutamate enhanced the activity by 35%, even though glutamate did not remove the sigmoidicity from the titration curve nor alter the total amount of DNA replicated (Fig. 2). This latter result indicated that

**Fig. 1. Chloride and glutamate effects on whole reaction kinetics.** Whole reaction kinetics were performed as described under "Experimental Procedures" using 26 units of holoenzyme. A, potassium chloride was added to the reaction in the following final concentrations: no added salt (○); 50 mM (●); 100 mM (△); and 150 mM (□). B, potassium glutamate was added in the following concentrations: no added salt (○); 50 mM (●); 250 mM (△); 500 mM (□); and 1 M (▲). C, replot of the DNA replicated at 5 min to show the chloride (○) and glutamate (●) effect on holoenzyme under the standard assay conditions. 100% DNA replicated was equivalent to 430 pmol of DNA replicated.

**Fig. 2. Holoenzyme activity and enhancement by 100 mM glutamate.** DNA synthesis was measured with various dilutions of holoenzyme as described under "Experimental Procedures" in a 5-min incubation with either no added salt (○) or with 100 mM potassium glutamate (●).
glutamate did not increase the activity of holoenzyme by increasing the amount of DNA capable of being replicated, in keeping with previous observations that anions do not interact with the DNA template (von Hippel and Schleich, 1969, and references cited therein). Despite several attempts to obtain a greater amount of replicated DNA, the observed maximum was never more than 440 pmol indicating that only about 80% of the total DNA was replicated. The remaining nonreplicable DNA can be accounted for by the presence of linearized DNA in the DNA preparation (Johanson and McHenry, 1982). Single-stranded DNA circles linearized by random nicks generate G4 origins located at random locations along the chain, resulting in only half the linear DNA being replicated. Gel electrophoresis of the template indicated that the ssDNA preparation contained about 70% circular DNA, 30% linear DNA (data not shown), consistent with a maximum of 80% total DNA replication.

Additive Salt Effects of Chloride and Glutamate—To determine whether there was a strong salt-specific binding site to which chloride bound and inhibited holoenzyme activity, we performed the assays in the presence of both chloride and glutamate. At four glutamate concentrations, 0, 50, 400, and 800 mM, a range of chloride concentrations were tested (Fig. 3). In the absence of glutamate, the chloride titration resembled that in Fig. 1C with about a 20% activity enhancement at low salt and 60% inhibition at 135 mM chloride. In 50 mM glutamate, no additional chloride was required to reach the 20% enhancement and a lower amount of chloride caused inhibition. Glutamate was not able to relieve chloride inhibition but instead its effect was additive with chloride. As glutamate was increased to higher concentrations, chloride inhibition was shifted to even lower concentrations. Since both of the salt effects caused by chloride and glutamate were additive, we concluded that glutamate did not contain chloride-specific binding sites that lead to inhibition. Instead, holoenzyme only contained general anion binding sites.

Anion Specificity of Mg²⁺ Enhancement and Inhibition—Holoenzyme, like all DNA polymerases, requires Mg²⁺ for activity. Titration of the holoenzyme assay with Mg²⁺ (Fig. 4) generated curves that had distinct anion-specific optimum concentrations. From 0 to 7.5 mM, both MgCl₂ and Mg(acetate)₂ titrations sigmoidally increased the replicative activity. It was above 7.5 mM that the anion-specific effects were seen. MgCl₂ had its optimum effect at about 10 mM and required 27 mM to reduce the maximum activity by half. Mg(acetate)₂ had its optimum effect from 12 to 20 mM and required 60 mM to reduce activity by half. Because the acetate anion caused inhibition at higher concentrations, its Mg²⁺ optimum resulted in a greater amount of total replicated DNA. When the molar amount of inhibitory chloride or acetate was compared in the potassium and magnesium titrations, the values were not coincident (data not shown) as would have been predicted by chloride- or acetate-specific inhibition constants. These data provided another indication that the effect was not due to a chloride- or acetate-specific inhibition but rather to a general anion phenomenon.

Elongation Kinetics: Two- and Three-step Reactions—Since the salt sensitivity of the holoenzyme assay included the effects from primase binding, primer synthesis, holoenzyme binding, and elongation kinetics, we eliminated the complications of the two primase steps by studying the elongation kinetics following initiation complex formation. However, there were two methods of forming initiation complex and we found that the method chosen influenced the subsequent elongation kinetics. In the three-step reaction (Fig. 5), primer was synthesized in the first step, initiation complex was formed in the second step, and elongation kinetics were monitored in the third step. The two-step reaction (Fig. 6) differed in that primer was synthesized and initiation complex formed concomitantly in the first step; these conditions for forming initiation complex mimicked more closely those in the holoenzyme assay. Regardless of the method used for complex formation, biphasic elongation kinetics were observed at all salt concentrations: a rapid burst due to synchronous DNA replication and a much slower second phase. The slower phase could be due to DNA polymerase IIIᵃ, the subassembly of holoenzyme that lacks the β subunit and exhibits much slower kinetics.

In the three-step reaction (Fig. 5A) with no added salt and limiting holoenzyme, 53% of the available circles were replicated in a burst of 35 s. The rate of elongation was (8600 nucleotides/35 s) = 246 nucleotides/s under these conditions. Adding chloride decreased the elongation rate (Fig. 5B); 200 mM chloride reduced the rate by half. Adding 50 mM chloride increased the number of replicated circles showing that low salt enhanced holoenzyme binding to DNA. Further increases of chloride reduced the number of replicated circles indicating that the holoenzyme-DNA binding was salt-dependent, thus
providing a basis for the salt dependence of the holoenzyme reaction.

In the two-step reaction (Fig. 6) in the absence of added salt, 57 ± 3% of the available templates were replicated in a burst of 26 ± 2 s. This was nearly the same amount of DNA replicated in the three-step reaction but the elongation rate was faster. The chloride dependence of DNA replication was nearly the same whether the initiation complex was formed by the two- or three-step method. When glutamate was substituted for chloride, the range of salt concentrations over which DNA was replicated was extended to much higher salt concentrations; the concentrations of added salt which reduced the DNA replicated to half-maximum were 120 mM chloride and 950 mM glutamate. In contrast, the salt sensitivity of the elongation rate was independent of the species of salt used; about 480 mM salt reduced the elongation rate by half. Thus the faster elongation rate of the two-step reaction was less salt sensitive than that of the three-step reaction.

Salt Sensitivity of DNA Polymerase III—The salt sensitivity of the DNA polymerase III activity (Fig. 7) contrasted sharply with that of holoenzyme. DNA polymerase III is the smallest subassembly of holoenzyme containing the catalytic polymerase subunit, the proofreading exonuclease subunit, and a third subunit of unknown function. Since DNA polymerase III was so much less processive than holoenzyme (Fay et al., 1981), its activity was determined in a gap-filling assay rather than on primed M13Gori ssDNA. Core activity was reduced to 50% maximum by 23 mM chloride, 80 mM acetate (data not shown), or 133 mM glutamate. Even though anion inhibition followed the same ranking as for holoenzyme, much less salt was required and there was no low salt enhancement. The extreme chloride sensitivity of DNA polymerase III activity has been noted before (Kornberg and Gefer, 1972; Otto et al., 1973; Livington et al., 1975; LaDuca et al., 1986).

Salt-enhanced Holoenzyme Thermal Stability—Diluted holoenzyme did not lose activity when preincubated at 0 or 30 °C but lost half its activity when preincubated at 42.0 °C (Fig. 8). The addition of chloride or glutamate to the preincubation increased the temperature of half-maximum activity to 45.0 and 46.5 °C respectively. When salt was present in the preincubation mixture, there was 8 mM final salt in the holoenzyme assay that resulted in a 32% enhancement of holoenzyme activity, as predicted in Figs. 1C and 2. That holoenzyme remained functional after preincubation with 200 mM chloride.

Fig. 5. Elongation kinetics in the three-step reaction. A, primed template was synthesized in the first step, initiation complex was formed with 113 units of holoenzyme and ATP in the second step, and the elongation kinetics were assayed in the third step at the indicated salt concentrations as described under "Experimental Procedures." The added KCl concentrations were none (●), 50 mM (○), 100 mM (×), and 200 mM (■). B, secondary plot of the elongation rate (●) and percent (total) DNA replicated (■) as determined from the burst of synthesis. The elongation rate was calculated assuming that the full DNA circle of 8623 nucleotides was replicated. The amount of DNA replicated in the burst was corrected for the slower second phase by extrapolating the secondary phase to the zero time point and obtaining the amount from the ordinate. 100% DNA replicated was equivalent to 430 pmol of DNA replicated.

Fig. 6. Elongation kinetics in the two-step reaction. Primer was synthesized and initiation complex was formed with 113 units of holoenzyme in a single step, and then elongation kinetics were measured at the indicated salt concentrations as described under "Experimental Procedures." Elongation rate versus chloride (○) or glutamate (■) and percent (total) DNA replicated versus chloride (□) or glutamate (■) were determined as described in the legend to Fig. 6.

Fig. 7. Salt titration of DNA polymerase III. Reaction was as described under "Experimental Procedures" with chloride (○) or glutamate (■) as indicated. 100% DNA replicated was equivalent to 430 pmol of DNA replicated.
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Fig. 8. Thermal stability of holoenzyme activity in the presence or absence of added salts. Holoenzyme diluted 1:20 into enzyme dilution buffer containing no salt (●), 200 mM chloride (O), or 200 mM glutamate (×) was incubated for 5 min at the indicated temperatures. The solution was then cooled on ice and 25 units assayed in the holoenzyme assay for 5 min at 30 °C as described under "Experimental Procedures." 100% DNA replicated was equivalent to 430 pmol of DNA replicated.

also showed that the inhibition seen in Fig. 1C was not due to an irreversible reaction.

**DISCUSSION**

Most biochemical observations concerning DNA polymerase III holoenzyme are consistent with it being responsible for the majority of E. coli replicative DNA synthesis. For instance, its dimeric nature allows for the coordination of leading and lagging strand polymerase activities while its very high processivity compensates for its low intracellular abundance (McHenry, 1988a). However, the enzyme's in vitro salt sensitivity has not been reconciled with the ability of E. coli to grow under high salt conditions. A resolution was suggested by work that established glutamate as the major free intracellular anion (Richey et al., 1987) and by work that showed that protein-DNA interactions could tolerate much higher glutamate concentrations than chloride (Leirmo et al., 1987; Overman et al., 1988). We show here that chloride and glutamate compete for general anion binding sites on holoenzyme and that the inhibition caused by these anions was the result of a change in the number of holoenzyme molecules bound to the template.

Salt inhibition of holoenzyme activity may result from any of a number of salt phenomena each with its own molecular interpretation: (i) an ion could bind to a particular site on holoenzyme and reduce the effectiveness of the active site; (ii) ionic strength could shield the protein and DNA electrostatic charges thereby lowering the binding affinity; or (iii) anions loosely bound at the protein-water interface could influence protein binding to the DNA. As in other studies on protein-DNA interactions, our results were consistent with the third possibility since our anion effect followed the Hofmeister series. In addition, we gained proof against the first and second possibilities.

The additive salt effects of chloride and glutamate indicated that there were not chloride-binding sites (or glutamate-binding sites) as in (i) above, but that there were general anion-binding sites. This was also demonstrated by the non-coincidence of the molar amount of inhibitory chloride (or acetate) in the potassium and magnesium titrations; chloride- or acetate-specific inhibition would predict mass action behavior by these anions. When the salt concentrations which caused 50% inhibition were adjusted for ionic strength, the salts retained their relative ranking: glycine (0 mM) < chloride (110 mM) ≈ phosphate (113 mM) < acetate (250 mM) < N-Ac-glutamate (435 mM) < aspartate (720 mM) < glutamate (1 M). If this had been an ionic strength effect as in (ii) above, the salt concentrations would have coincided.

Low concentrations of potassium salts stimulated holoenzyme activity. The stimulation did not alter the total percent DNA replicated indicating that the effect was strictly on the protein. We have shown that the presence of salt leads to increased thermal stability of holoenzyme and that this may account in part for the enhancement which results from low added salt.

At moderate salt concentrations (100 mM to 1 M) there was inhibition of holoenzyme activity which was anion-specific. The anion inhibition of holoenzyme followed the Hofmeister series: chloride > acetate > glutamate. The Hofmeister series is an empirical ranking of anions based on their effects on protein solubility (Hofmeister, 1888) although the same ranking has been observed in a great number of different systems (see Collins and Washabaugh, 1985, for a detailed list). These ion-specific effects arise from the manners in which various ions interact with an interface (Collins and Washabaugh, 1985), in our case a protein-water interface. Ions that interact with a protein interface more strongly than bulk water tend to stabilize protein structure. When comparing the classical ranking (SO$_4^-$ ≈ HPO$_4^{2-}$ > F$^-$ > Cl$^-$ > Br$^-$ > I$^-$ > ClO$_4^-$ > SCN$^-$) with that obtained in recent protein-DNA studies (glutamate$^-$ > F$^-$ > acetate$^-$ > Cl$^-$ > Br$^-$ ≈ NO$_3^-$ > I$^-$ > ClO$_4^-$) one can see that phosphate should produce an effect on the order of glutamate. However, we found that phosphate ranked lower than chloride in its effect on the holoenzyme assay. A simple explanation for this anomalous behavior, that we favor, is that phosphate inhibits holoenzyme by a mechanism different from the other anions; there is probably a phosphate-specific binding site on holoenzyme that interferes with the active site.

Other anions not ranked in the usual Hofmeister series were tested in an attempt to discern which glutamate moieties provided its benevolent properties. Aspartate was second only to glutamate in extending the salt concentration range over which holoenzyme was functional. Thus the methylene difference between glutamate and aspartate resulted in significant but not extreme differences between these two anions. N-Ac-glutamate has the α-amino functional group of glutamate blocked resulting in a dicarboxylic acid and, even though N-Ac-glutamate ranked higher than chloride, it was less effective than the monocarboxylic acid, acetate. Another indication of the overriding importance of the α-amino functional group was that glycine, which lacks the carboxyl side chain of glutamate and aspartate, was of intermediate effect between acetate and aspartate. It appeared that it was the juxtaposition of the amino and carboxyl groups which provided the largest relative contribution of the moieties to glutamate's ranking in the Hofmeister series. Zwitterionic amino acids can potentially neutralize both positive and negative charges in a manner that would decentralize the charge distribution while doing so. Likewise, Collins and Washabaugh (1985) proposed that one of the functions of bound anions was to distribute the charge transferred among several adjacent water molecules.

The salt sensitivity of the whole reaction resulted from the combined sensitivities of primase and holoenzyme. To study the salt sensitivity of holoenzyme-DNA binding independent from primase activity we formed the initiation complex. There were two methods of forming initiation complex which re-
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<table>
<thead>
<tr>
<th>Primer, template, and how initiation complex formed</th>
<th>Time</th>
<th>Rate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primase-primed G4 in presence of holoenzyme</td>
<td>±15</td>
<td>±370</td>
<td>Johanson and McHenry, 1982</td>
</tr>
<tr>
<td>Primase-primed G4, then holoenzyme added</td>
<td>18</td>
<td>310</td>
<td>Burgers and Kornberg, 1983</td>
</tr>
<tr>
<td>15-mer DNA-primed M13Gori, then holoenzyme added</td>
<td>~39^a</td>
<td>~372^b</td>
<td>O'Donnell and Kornberg, 1985</td>
</tr>
<tr>
<td>Primase-primed M13Gori, then holoenzyme added</td>
<td>Average 30, 15^b</td>
<td>Average 287, 575^b</td>
<td>Maki and Kornberg, 1988b</td>
</tr>
<tr>
<td>Primase-primed M13Gori in presence of holoenzyme</td>
<td>35</td>
<td>250</td>
<td>Fig. 5</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>330</td>
<td>Fig. 6</td>
</tr>
</tbody>
</table>

*Elongation times were estimated from the referenced data and the rates (nucleotides/s) were calculated from the number of replicated nucleotides divided by the elongation time; G4 is 5577 nucleotides and M13Gori is 8623 nucleotides.

Rate was for the fastest holoenzyme in the assay; the average rate was somewhat slower.

M13Gori was linearized and only ~7600 nucleotides replicated.

The salt effect on DNA replicated during burst synthesis strongly resembled that for the holoenzyme reaction with low salt enhancement and high salt inhibition. From the burst data we concluded that high salt inhibition was due to a reduction in the number of initiation complexes prior to the start of elongation. Holoenzyme has such a high processivity that the entire length of the relatively short template used in our assay was easily replicated in a single elongation event. The chloride sensitivity was nearly identical in the two- and three-step reactions indicating that holoenzyme-template binding was not sensitive to the formation method.

There have been numerous studies dealing with initiation complex formation and the subsequent burst of synchronous DNA elongation that follows dNTP addition. A comparison of published elongation rates indicates that the average elongation rate has remained roughly the same despite assays using different buffer systems and methods of quantification (see Table I for selected references). Unexpectedly, we found that the method of forming the initiation complex had a significant influence on the subsequent elongation rate, differing in magnitude and salt sensitivity. In the three-step reaction, the elongation rate was 250 nucleotides/s and was 50% reduced by 200 mM chloride. The elongation rate in the two-step reaction was 330 nucleotides/s and was 50% reduced by 480 mM salt.

The dramatic difference in elongation rates resulted from the manner in which primase was incorporated into the initiation complex. Presumably, a more natural complex was formed when primase was incorporated during active primer synthesis as in the two-step reaction and in the one-step holoenzyme assay. The resulting elongation rate was faster and less sensitive to salt than when holoenzyme was added after the completion of primer synthesis as in the three-step reaction. This indicated that when holoenzyme incorporated active primase into initiation complex, the complex so formed was more efficient.

A full understanding of the molecular interactions of a natural replicative complex has to include the underappreciated role of salt effects. Holoenzyme remained active at much higher in vitro salt concentrations when glutamate rather than chloride was the anion. E. coli apparently makes use of this intrinsic anion difference by using glutamate as its primary intracellular osmolytic anion (Richey et al., 1987). When grown in media containing 1 M NaCl, the concentration of intracellular glutamate is raised to 600 mM, at which concentration we have found that holoenzyme was almost fully functional. In contrast, 200 mM chloride completely inhibited holoenzyme activity. Thus, glutamate overcomes the salt inhibition of holoenzyme. Future studies of holoenzyme and its components should be conducted in the presence of glutamate given the profound influence that this anion has upon the functional properties of the natural replicative complex.

REFERENCES


Hofmeister, F. (1888) Naunyn-Schmiedebergs Arch. Exp. Pathol. Pharmak. 24, 247-260


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