DIFFERENTIAL *N*-ETHYLMALEIMIDE INHIBITION OF TWO ENZYMES OF THE DNA α-POLYMERASE-FRACTION FROM CALF THYMUS

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1. Introduction

The DNA α-polymerase (DNA-nucleotidyltransferase: EC 2.7.7.7.) fraction of calf thymus has been shown to be heterogeneous [1–3]. Using DEAE-cellulose chromatography, most preparations can be resolved into three enzymes A_1, A_2 (both sedimenting at 8.0–8.4 S) and C (7.3 S), detected by means of an activated DNA template. In some preparations a further enzyme, B (5.2 S), is found and probably arises proteolytically from enzyme C [2,4]. A fourth species of enzyme, D, has also been detected by its response to the synthetic template poly(dA) . $(dT)_{10}$. Evidence has been presented that enzyme C (mol. wt $150–170 \times 10^3$) can be derived in vitro from the A enzymes (mol. wt $200–230 \times 10^3$) by exposure to 2.4 M urea. Whether this treatment eliminates a true sub-unit or a pre-existing fragment created by proteolytic cleavage of the A enzymes either during preparation or in vitro is still not clear [5]. The additional components is evidently fairly basic in character [6].

The sensitivity of DNA α-polymerase to sulphydryl reagents is commonly used as a criterion to distinguish it from the much less sensitive β-polymerase [7–9]. In this paper, however, it is shown that considerable differences exist in the reactivities of α-polymerase enzymes A and C to inhibition by *N*-ethylmaleimide (NEM) and to the protective effects of the various substrates.

Abbreviations: NEM, *N*-ethylmaleimide; dNTP, deoxyribonucleoside 5′-triphosphate

2. Materials and methods

N-ethylmaleimide (NEM) was purchased from Sigma. Activated calf thymus DNA was prepared as described by Loeb [10].

Calf thymus DNA α-polymerase was prepared and resolved into its subfractions by DEAE-cellulose chromatography as previously described [2]. In the present studies enzymes A_1 (referred to in the text as enzyme A) and C were taken and chromatographed a second time on DEAE-cellulose before use (specific activities were 1300 and 2300 units/mg protein, respectively). Although enzyme A was freed of enzyme C, it did contain varying amounts of enzyme A_2 from which, other than by DEAE-cellulose chromatography, it is indistinguishable [5].

2.1. *Inhibition reactions*

Incubations with NEM were carried out in 0.1 ml reaction mixtures containing 25 mM phosphate buffer, pH 7.8, 16% (w/v) glycerol, 0.1 mM dithiothreitol (except in fig.1), the indicated level of NEM and enzyme. Inclusion of a low level of dithiothreitol reduced the otherwise unacceptably high rates of enzyme activity loss in controls (up to 40% in 1 h). Incubation of enzymes A and C with 0.25 mM NEM in the presence of dithiothreitol, produced the same degree of inhibition at all levels from 0 to 0.1 mM. NEM, therefore, reacted faster with enzyme than with added thiol. Reactions were carried out at the temperatures indicated and 10 μl aliquots taken at various times for assay of polymerase activity in final volumes of 0.125 ml. Under the conditions of the assay (containing 1 mM dithiothreitol), incorpora-

tion of [^{3}H]dTMP was linear with time for at least 20 min.

The standard DNA polymerase assay using activated DNA as template was performed as described [2,11]. For convenience in time courses, assay incubation time was 11 min.

3. Results and discussion

3.1. *Differential inhibition of enzymes A and C by NEM*

In preliminary incubations carried out at 25°C for 30 min with increasing NEM levels, it was found that while inhibition of enzyme A was about 60% with 1 mM NEM, enzyme C was almost totally inactivated. The difference in sensitivity of enzymes A and C of particular interest as a means of investigating further the relationship between them.

Figure 1 compares the time courses of the inhibition of enzymes A and C by 0.125, 0.25 and 1 mM NEM. For enzyme C an initial fast inactivation corresponding to about 50% loss of enzyme activity, occurred in the first 10 min and was followed by a slow phase showing pseudo first-order kinetics. These results imply the presence of at least two main classes of thiols (assuming the reaction of these

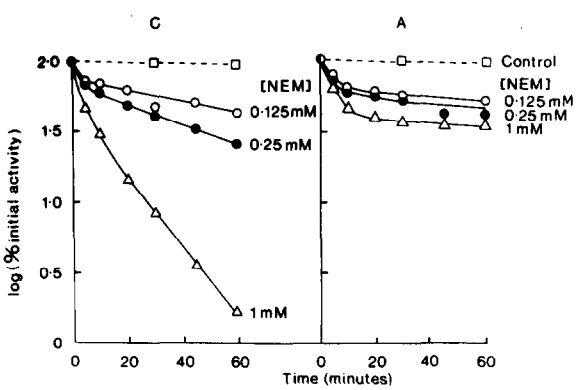

Fig.1. Inhibition of DNA polymerases A and C by NEM. Enzymes C (left panel) and A (right panel) were incubated with the indicated levels of NEM in the presence of 0.01 mM DTT. Incubations were carried out at 0°C in order to obtain accurate curves in the presence of 1 mM NEM. 100% activity was 333 pmol and 428 pmol [^{3}H]dTMP incorporated in 11 min by enzymes A and C, respectively, in the standard assay.

groups alone). The pseudo first-order rate constants for the slow phase thiols, 9×10^{-3}, 16×10^{-3} and 55×10^{-3} min^{-1}, were essentially a linear function of the three NEM concentrations used and for enzyme C this phase is, therefore, first-order in NEM. For enzyme A only the fast set of thiols were particularly evident, the second phase proceeding at a much lower rate and showing zero-order kinetics with respect to NEM (6×10^{-3} min^{-1} at all three NEM concentrations). The amplitude of the fast phase of the reaction increases with NEM concentration but not in a proportional way; it is probably maximal with 1 mM NEM although the assay methods used make accurate analysis of the fast phase difficult.

The data indicate that the fast reacting set of thiols are required for maximal activity since their alkylation reduces the α-polymerase activity, as in enzyme A, but does not totally eliminate it. Conversely, reaction of the slow set of thiols with NEM completely abolishes activity as in enzyme C. Either access by NEM to these essential thiols is limited by some structural feature in enzyme A or their reactivity is modified in some way. This is likely to be a function of the additional component present in enzyme A.

Incubation of enzymes A and C with 0.25 mM NEM, carried out in a buffer of ionic strength of about 0.5, sufficient to dissociate aggregates [12], still showed the characteristic differences in NEM sensitivity seen in fig.1. Therefore, the aggregation of enzyme A is not responsible for its limited susceptibility to NEM. Further studies showed that, following partial inactivation of enzyme A by incubation for 30 min with 0.25 mM NEM, all of the residual activity chromatographed on DEAE-cellulose precisely in the position of the unreacted enzyme A (recovery for the column was 90–100%). The re-isolated, partially inactivated enzyme was, as expected, less sensitive to NEM than the original untreated enzyme.

3.2. *Protection experiments*

Both enzymes are protected against NEM inhibition by DNA (fig.2). DNA subjected to limited digestion by deoxyribonuclease I to expose 3′-OH groups (activated DNA) afforded the best protection. Denatured DNA was less effective than activated DNA and native DNA is only slightly protective. In

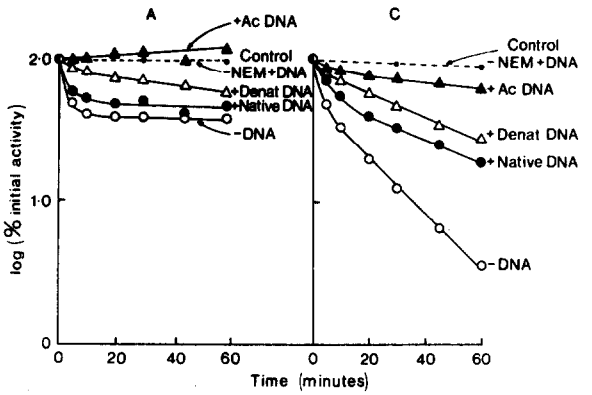

Fig.2. Protection by DNA against inhibition by 0.25 mM NEM. Enzymes A (left panel) and C (right panel) were incubated with 0.25 mM NEM at 22°C in the presence of 0.1 mM DTT. DNA was employed at 50 μg/ml. The activated DNA level in the assay was adjusted accordingly in the relevant case. 100% activity was 128 pmol and 194 pmol [^{3}H]dTMP incorporated by enzymes A and C, respectively.

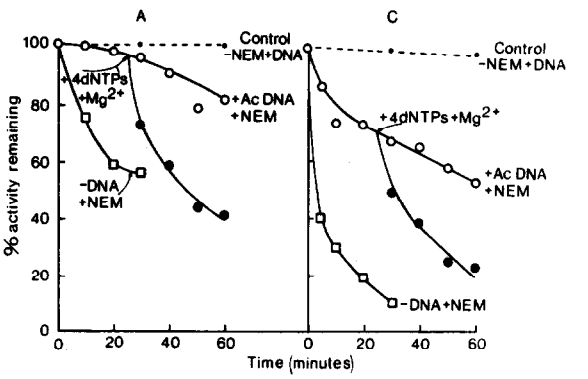

Fig.3. Reversal of protection by DNA on adding 4 dNTPs and Mg^{2+} together. Enzymes A (left panel) and C (right panel) were incubated at 22°C with 0.25 mM NEM and 0.1 mM DTT. Activated DNA concentration was 50 μg/ml. The 4 dNTPs (0.1 mM each, final concn.) plus Mg^{2+} (10 mM) were added simultaneously at the points indicated. 100% activity was 158 pmol and 147 pmol [^{3}H]-incorporated for A and C, respectively. Results are plotted directly as percentages to emphasize the effects of the additions.

further experiments maximum protection of enzyme A was achieved by activated DNA at 50 μg/ml but protection of enzyme C was only 80% of the control value with this level of DNA. Further addition of DNA up to 300 μg/ml increased this to 87%. Since enzyme A exhibits mainly a single phase of NEM inactivation, this suggests that the fast reacting thiols are those most readily protected by the 3′-OH groups of activated DNA in both A and C, while the slow set are more difficult to protect as in enzyme C.

In further experiments, addition of Mg^{2+} alone or of Mg^{2+} plus dNTPs, offered no protection to either enzyme against NEM inhibition; the results were identical to those in fig.1. On the other hand, addition of Mg^{2+} plus dNTPs together to enzymes A or C, to which activated DNA and NEM had already been added (in that order), resulted in an immediate reversal of the protection for both enzymes (fig.3). These additions complete the requirements for DNA synthesis and suggest that when the polymerase is induced to copy the template it becomes prone to inactivation. This is confirmed by experiments in which 0.25 mM NEM is added to incubations carried out under normal assay conditions (except that only 0.01 mM dithiothreitol was present); in the control, DNA synthesis proceeds linearly for 20 min. while in a portion to which NEM is added at 6 min., it ceases despite the presence of activated DNA (fig.4). These results are explicable in terms of the distributive behaviour of DNA polymerase molecules in

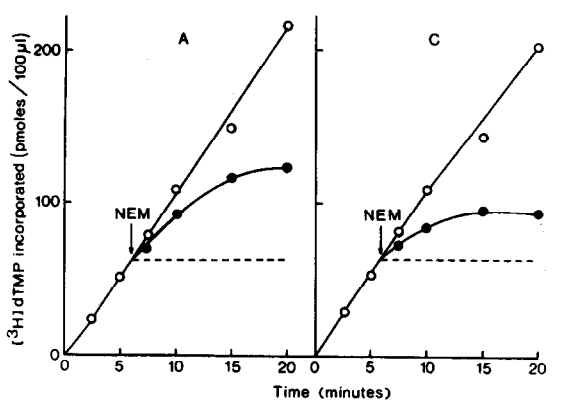

Fig.4. Inhibition of enzymes A and C in complete assay mixtures by 0.25 mM NEM 1.25 ml DNA polymerase assays (containing 0.01 mM DTT) of enzyme A (left panel) and C (right panel) were incubated at 37°C. Acid precipitable radioactivity in 0.1 ml aliquots was determined at the indicated times. At 6 min, 0.5 ml was removed from each incubation and made 0.25 mM in NEM. (o——o) Control, (•——•) + NEM.

in vitro system [13]; the released molecules becoming susceptible to inhibition by NEM.

However, a more interesting observation was made when Mg^{2+} was added, in the absence of dNTPs, to enzyme A previously incubated in the presence of activated DNA and NEM (added in that order). This addition of Mg^{2+} now led to a complete reversal of the protection by DNA of the fast set of thiols (fig.5 left panel). By contrast, addition of Mg^{2+} to a similar incubation mixture containing enzyme C led to a relatively small increase in the rate of inhibition (fig.5 right panel). The further addition of dNTPs, thereby completing DNA synthesis conditions, caused a marked enhancement of thiol reactivity in the case of enzyme C whereas the effect was slight for enzyme A. (Addition of dNTPs alone to DNA-protected enzyme was without effect.)

The addition of Mg^{2+} to enzyme A in the presence of activated DNA results therefore in either a conformational change in the enzyme or the destabilising of the DNA–enzyme complex, leading to re-exposure of the fast-reacting thiols. The effect on enzyme C in the same situation is much less dramatic, presumably because it lacks the additional structural component present in A. The data can be explained in two other ways. First, that nuclease activity might be associated with the additional component of enzyme A and that this attacks the protecting DNA on addition of Mg^{2+}. This is unlikely since the reversal of protection on addition of Mg^{2+} is as rapid and as complete at 0°C as at 22°C. A second possibility is that results with Mg^{2+} arise because enzyme A preferentially finds dNTPs throughout several stages of purification using cation and anion-exchange resins, gel-filtration at high ionic strength, etc. We have no proof of this but think that this is also unlikely.

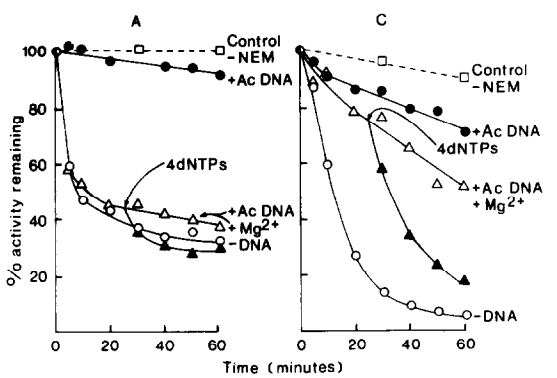

Fig.5. Reversal of protection by DNA when Mg^{2+} and 4 dNTPs are added successively. Enzymes A (left panel) and C (right panel) were incubated at 22°C with 0.125 mM NEM in the presence of 0.1 mM DTT. Activated DNA (50 μg/ml), 10 mM Mg^{2+} and 0.1 mM of each of the four dNTPs were added as indicated. 100% activity was 126 pmol and 148 pmol [^{3}H]dTMPs incorporated by enzymes A and C respectively.

It is apparent that further analysis of the effects reported here will require a more detailed study of the interaction between the primer 3′-hydroxyl of DNA and the fast set of thiols.

Finally, the additional component in enzyme A is probably also responsible for its heat stability compared with enzyme C [5].

Acknowledgement

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