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The Observed Inhibitory Potency of 3′-Azido-3′-deoxythymidine 5′-Triphosphate for HIV-1 Reverse Transcriptase Depends on the Length of the Poly(rA) Region of the Template†

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ABSTRACT: The inhibitory potency of 3′-azido-3′-deoxythymidine 5′-triphosphate (AZTTP) against HIV-1 reverse transriptase (HIV-1 RT) has been further evaluated. The results indicate that the previously reported low K_i values for AZTTP against HIV-1 RT (2–35 nM) are due neither to the direct tight binding of AZTTP to HIV-1 RT nor to the interaction of the enzyme with AZTMP moiety terminated primer-templates, but instead they are an artifact of the use of a homotemplate-primer [poly(rA)·oligo(dT)]. With a set of RNAs of defined sequence as templates, we demonstrate that the observed K_i value for AZTTP depends on the length of the poly(rA) region following the primer in the RNA template. The more adenosyl residues in the RNA template that are available for processive incorporation of TMP moieties, the lower is the observed K_i value for AZTTP. Since the potencies of new inhibitors of HIV-1 RT are usually compared with that for AZTTP, these results have important consequences for the process of discovery of new HIV inhibitors that are of potential use in AIDS therapy.

3′-Azido-3′-deoxythymidine (AZT),[1] the first drug used clinically for the treatment of human immunodeficiency virus (HIV) infection, is considered to be a prodrug that is converted into 3′-azido-3′-deoxythymidine 5′-triphosphate (AZTTP) by cellular kinases (Mitsuya et al., 1985; Fischl et al., 1987; Furman et al., 1986). In vitro kinetic studies show that AZTTP is a very potent competitive inhibitor of HIV-1 RT with observed K_i values ranging from 2 to 35 nM (Furman et al., 1986; Kedar et al., 1990; Reardon et al., 1990; Eriksson et al., 1989; Vrang et al., 1987, 1988; Cheng et al., 1987; St. Clair et al., 1987; Ono et al., 1986; Matthes et al., 1987; White et al., 1989; Starnes et al., 1989), and the therapeutic effect of AZT toward HIV infection has been attributed to this inhibition (Eriksson et al., 1989; Vrang et al., 1987; Cheng et al., 1987).

The inhibitory mechanism of AZTTP has been studied extensively. It was demonstrated that AZTTP is a substrate for HIV-1 RT (Kedar et al., 1990; Reardon & Miller, 1990)

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[1] Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZTTP, 3′-azido-3′-deoxythymidine 5′-triphosphate; DE, (diethylamino)ethyl-cellulose; EDTA, ethylenediaminetetraacetic acid; HIV-1 RT, human immunodeficiency virus 1 reverse transcriptase; NMP, nucleoside 5′-monophosphate; TMP, thymidine 5′-monophosphate.

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and that the incorporation of an AZTMP moiety into the DNA chain results in DNA chain termination. Since AZTTP is a potent inhibitor with an observed K_i value many orders of magnitude lower than the K_d value for TTP (K_d for TTP is about 36.7 μM; Painter et al., 1991), it is unknown whether the inhibitory potency arises from chain termination alone or if the 3′-azido substitution contributes to the inhibitory potency of AZTTP at an earlier stage in the enzymatic process.

One possible contribution to the inhibitory potency of AZTTP is that the azido group of AZTTP makes it bind more tightly to HIV-1 RT than does TTP itself in the Michaelis complex. It has been reported that AZTTP has a K_d value of 47.3 μM, which is similar to that for TTP (K_d for TTP = 36.7 μM) (Painter et al., 1991). These experiments suggest that AZTTP actually binds to HIV-1 RT somewhat more weakly than TTP. A similar conclusion was also made from kinetic studies using nonhydrolyzable α,β-imido analogues of TTP and AZTTP (Ma, 1991).

Another possible contribution to the inhibitory potency is that the terminated primer-template has a higher affinity to HIV-1 RT than the normal primer-template. Precedence for this possibility was found in the inhibition of DNA polymerase I by 2′,3′-anhydroadenosine 5′-triphosphate (Catalano & Benkovic, 1989), which is also a substrate for the enzyme. The 2′,3′-anhydroAMP-terminated primer-template forms a dead-end complex with DNA polymerase I and, overall, renders 2′,3′-anhydroadenosine 5′-triphosphate a potent inhibitor for the enzyme. Although the dissociation of HIV-1 RT from the normal primer-template and succinylfluorescein-labeled ddTMP-terminated primer-template has been studied (Müller et al., 1991), we are still not clear about whether AZTTP has an effect on HIV-1 RT similar to that of 2′,3′-anhydroadenosine 5′-triphosphate on DNA polymerase I.

The present paper describes our studies on the contribution of 3′-azido substitution to the inhibitory potency of AZTTP. The results have led to a finding that the low observed K_i values for AZTTP against HIV-1 RT are artifacts of using homopolynucleotides as templates.

Materials and Methods

The in vitro transcription kit was from Promega; poly(rA), $(dT)_{14}$, oligo$(dT)_{12-18}$·poly(rA), DNA polymerase I large fragment (Klenow), T4 polynucleotide kinase, and four deoxyribonucleoside 5′-triphosphates (dATP, dGTP, dCTP, dTTP) were from Pharmacia; HIV-1 reverse transcriptase (P66) was prepared as described previously (Bathurst et al., 1990). Other reagents were from Sigma.

Synthesis and Identification of 5′-^{32}P-3′-Azido$(dT)_{15}$. $(dT)_{14}$ (Pharmacia) was 5′-labeled with ^{32}P by T4 polynucleotide kinase according to the method recommended by the manufacturer (Pharmacia). The 5′-^{32}P$(dT)_{14}$ was then annealed with equimolar amounts of poly(rA), and the resulting primer-template was used to synthesize the needed oligonucleotide. The reaction mixture for the synthesis is the same as that described in the general method for HIV-1 RT inhibition assay except for the following: 1 μM poly(rA)·5′-^{32}P$(dT)_{14}$, 30 μM AZTTP, 0.3 μg/mL HIV-1 RT (P66), and no TTP. After incubation at 37 °C for 1.5 h, the reaction was quenched with EDTA and the needed oligonucleotide was purified by an anion-exchange column (NACS) PREPAC from Biochemical Research Laboratory) to remove unreacted AZTTP using the method recommended by the manufacturer. The oligodeoxynucleotide obtained was further purified by preparative 20% polyacrylamide/7 M urea gel and visualized by autoradiography, and the desired band was extracted by conventional methods (Ogden et al., 1987). The extracts were desalted by a Sep-Pak cartridge(Waters), characterized by electrophoresis (Figure 1a), and quantitated by radioactivity measurements.

Inhibition of HIV-1 RT by 5′-^{32}P-3′-Azido$(dT)_{15}$·Poly(rA). 5′-^{32}P-3′-Azido$(dT)_{15}$ was mixed with poly(rA) in the annealing buffer (10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$). The mixture was heated to 90 °C for 2 min, placed at 50 °C, and allowed to cool slowly over 30 min to form the terminated product, 5′-^{32}P-3′-azido$(dT)_{15}$·poly(rA). p$(dT)_{14}$ was also annealed with poly(rA) in the same manner to form the template-primer. The assay was the same as the general method for HIV-1 RT inhibition assay except that constant amounts of p$(dT)_{14}$·poly(rA) (0.5 μM) and [^{3}H]TTP (20 μM) and variable amounts of 5′-^{32}P-3′-azido$(dT)_{15}$·poly(rA) were used.

Percent Conversion of 5′-^{32}P$(dT)_{14}$·Poly(rA) to 5′-^{32}P-3′-Azido$(dT)_{15}$·Poly(rA) by 10 nM AZTTP. The reaction conditions were the same as in the experiments for inhibition of HIV-1 RT by 5′-^{32}P-3′-azido$(dT)_{15}$·poly(rA) except that both TTP and 5′-^{32}P-3′-azido$(dT)_{15}$·poly(rA) were absent and 10 nM AZTTP was present. The reaction was examined by autoradiography after electrophoresis on a 20% polyacrylamide/7 M urea gel. The intensities of the bands for 14-mers and 15-mers were scanned with a densitometer, and the values were used to calculate the percent conversion.

General Methods for HIV-1 RT Inhibition Assay. The assay mixture (20 μL) for the kinetic study contained 50 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, 8 mM DTT, 80 mM KCl, 0.4 μM poly(rA)·oligo(dT), 60 ng/mL of HIV-1 RT (P66), different primer-templates and inhibitors, and variable amounts of [^{32}P]TTP (4–20 μM, 0.2–1 μCi/20 μL). After incubation at 37 °C for 30 min, the mixtures were quenched by 5 μL of 0.5 M EDTA, and the primer-templates were collected on DE-81 paper. The incorporation rate of TMP moieties was measured by counting ^{32}P on the DE-81 paper, and the K_i values were calculated from double-reciprocal plots.

Preparation of RNA Templates with Different Lengths of the Poly(rA) Region. The RNA templates, $r(A)_1$, $r(A)_{10}$, and $r(A)_{50}$, were prepared from three sets of synthetic dsDNA fragments containing the following sequences: 3′-TCGACCGAATAGCTTTAATTATGCTGAGTGATATCCCTCTGGCCTTCGAACGTACGGACG(T)$_n$CCAGCTGAGATCTCCTAGGGG and 5′-AGCTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCGGAAGCTTGCATGCCTGC(A)$_n$GGTCGACTCTAGAGGATCCCC, where n = 1, 10, or 50. The positive strands of the DNAs were synthesized by Biomolecular Resource Center at UCSF, annealed with the primer described in the legends of Table I, and converted into dsDNA by Klenow fragment using conventional methods. Each of the dsDNA fragments was used as the template for T7 RNA polymerase in vitro transcription. The synthesis and purification of the RNAs were performed using protocols provided by the manufacturer (Promega), and the concentrations of the RNAs obtained were measured by the amount of [^{3}H]UMP incorporation. The primer-templates were prepared by annealing each RNA with equimolar amounts of synthetic oligodeoxynucleotide (d_{21}-mer) in the same way as that described in the section on the inhibition of HIV-1 RT by 5′-^{32}P-3′-azido$(dT)_{15}$·poly(rA). The resulting primer-templates were ready for kinetic studies.

Inhibition of HIV-1 RT by AZTTP with Different RNAs as Templates. The assay conditions are the same as those described in the general methods for the HIV-1 RT inhibition assay; 0.4 μM four different primer-templates [the primer-templates in the legends of Table I and poly(rA)·$(dT)_{12-18}$]

and variable amounts of AZTTP were used.

Results and Discussion

Inhibition of HIV-1 RT by 5′-^{32}P-3′-Azido(dT)$_{15}$·Poly(rA). It has been reported that oligonucleotides inhibit HIV-1 RT by competing at the active site of the enzyme with the normal primer-templates (Majumdar et al., 1988). These oligonucleotides usually do not bind better than the normal primer-templates unless they have special sequences (Majumdar et al., 1988, 1989). If 3′-azido substitution makes a contribution other than chain termination to the inhibitory potency of AZTTP by forming a 3′-azidothymidyl-terminated primer-template, a situation similar to the inhibition of DNA polymerase I by 2′,3′-anhydroadenosine 5′-triphosphate (Catalano et al., 1989) will occur, and the 3′-azidothymidyl-terminated primer-template should compete for HIV-1 RT more strongly than the normal primer-template; this effect can be studied by a competition experiment. We designed such an experiment using 5′-^{32}P-3′-azido(dT)$_{15}$·poly(rA) as a competitor and 5′-^{31}P(dT)$_{14}$·poly(rA) as the normal primer-template. Competition was observed by the decrease of [^{3}H]TMP moiety incorporation as the concentration of 5′-^{32}P-3′-azido(dT)$_{15}$·poly(rA) increased.

The purified 5′-^{32}P-3′-azido(dT)$_{15}$ showed a single band on a 20% polyacrylamide/7 M urea gel (Figure 1a). 5′-^{32}P-3′-Azido(dT)$_{15}$ was then annealed with poly(rA), and the terminated primer-template was used to compete with the normal primer-template, p(dT)$_{14}$·poly(rA). Figure 1b shows the results of the competition experiment. Fifty percent inhibition was obtained when the terminated primer-template reached about the same level as that of the normal primer-template. This experiment shows clearly that the 3′-azido-terminated primer-template does not bind to HIV-1 RT more tightly than the normal primer–template.

Further experiments showed that the inhibitory potency of AZTTP does not arise from the enzyme binding to the terminated primer-template which accumulated during assay. In the presence of 20 μM TTP, 10 nM AZTTP caused 50% inhibition in our assay system. Under the same conditions, but with no TTP present, 10 nM AZTTP can only convert 1% of 5′-^{32}P(dT)$_{14}$·poly(rA) into 5′-^{32}P-3′-azido(dT)$_{15}$·poly(rA). Since AZTTP can be incorporated into DNA no faster than TTP, in the presence of 20 μM TTP (conditions under which 10 nM AZTTP gave rise to 50% inhibition), 10 nM AZTTP would generate <1% of 5′-^{32}P-3′-azido(dT)$_{15}$·poly(rA). We conclude that the terminated primer-template accumulated during the assay would generate insignificant inhibition. The terminated primer-template accumulated during the assay is evidently not a major contributor to the potency of AZTTP.

Template-Length-Dependency Hypothesis. Our experiments and previous results (see introduction) suggest that 3′-azido substitution does not make a major contribution to the inhibitory potency of AZTTP, except for the fact that it obviously leads to chain termination after incorporation of an AZTMP moiety in the DNA chain. We have noted that the reported K_i values for AZTTP vary greatly. The lower K_i values (2–35 nM) were obtained from the experiments with oligo(dT)·poly(rA) as a primer-template (Furman et al., 1986; Kedar et al., 1990; Reardon et al., 1990; Eriksson et al., 1989; Vrang et al., 1987, 1988; Cheng et al., 1987; St. Clair et al., 1987, Ono et al., 1986; Matthes et al., 1987; White, et al., 1989; Starnes et al., 1989), whereas the higher K_i value (160 nM) reported by Reardon and Miller (1990) was obtained by using an RNA of defined sequence as template. These data suggest that, besides the conditions used in the experiments, the nature of a primer-template also plays a role in the observed inhibitory potency of AZTTP.

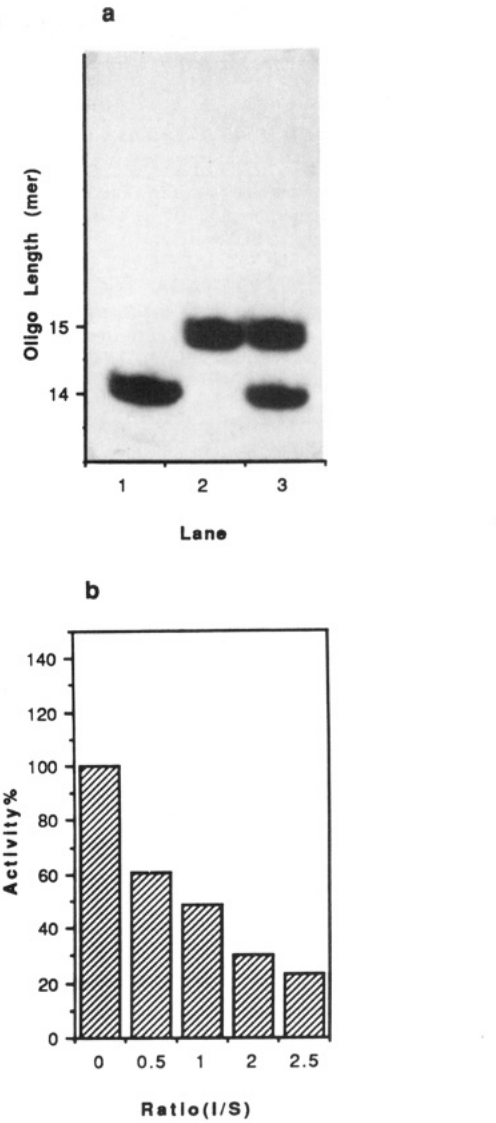

FIGURE 1: (a) Identification of 5′-^{32}P-3′-azido(dT)$_{15}$. The autoradiogram was obtained from a 20% polyacrylamide/7 M urea gel: lane 1,5′-^{32}P(dT)$_{14}$; lane 2, 5′-^{32}P-3′-azido(dT)$_{15}$; lane 3, 5′-^{32}P(dT)$_{14}$ + 5′-^{32}P-3′-azido(dT)$_{15}$. (b) Inhibition of HIV-1 RT by 5′-^{32}P-3′-azido(dT)$_{15}$. The I/S on the horizontal axis of the figure represents the molar ratio of 5′-^{32}P-3′-azido(dT)$_{15}$·poly(rA) to 5′-^{31}P(dT)$_{14}$·poly(rA).

Our hypothesis is that the length of the poly(rA) region of a template determines the observed inhibitory potency of AZTTP in vitro. In the assay system which gave lower observed K_i values, oligo(dT)·poly(rA) was used as a primer-template and [^{3}H]TTP was used as a substrate. The inhibition was measured by the decrease of incorporation of [^{3}H]TMP moieties when AZTTP was added into the assay mixture. Suppose we have two sets of templates: template A, which allows the incorporation of only one TMP moiety, and template B, which allows the incorporation of TMP moieties up to 100. Since the polymerization catalyzed by HIV-1 RT is a processive event, the results during the competitive incorporation of AZTMP and TMP moieties will be different with template A and template B. With template A, only one AZTMP or TMP moiety can be incorporated into the template (i.e., incorporation of one AZTMP moiety can only block incorporation of one TMP moiety). With template B, a TMP moiety can be processively incorporated into the template up to 100; if one AZTMP moiety is incorporated into the position im-

Table I: Observed K_i Values for AZTTP with Different RNAs as Templates[a]

primer-template[b]	K_i value (nM)	primer-template[b]	K_i value (nM)
$r(A)_1 \cdot d_{21}$	130 ± 12[c]	$r(A)_{50} \cdot d_{21}$	8.6 ± 0.9[e]
$r(A)_{10} \cdot d_{21}$	21 ± 4[d]	poly(rA)·$(dT)_{12-18}$	3.6 ± 0.4[f]

[a] The inhibition patterns for the experiments in this table are all competitive. [b] Except poly(rA)·$(dT)_{12-18}$, the primer-templates have the following structures: RNA template, 3′-CCCCUAGGAGAUCUCAGCUGG$(A)_n$CGUCCGUACGUUCGAAGGCCAGAGGG; DNA primer, 5′-GGGGATCCTCTAGAGTCGACC. For n = 1 primer-template is abbreviated as $r(A)_1 \cdot d_{21}$-mer; for n = 10, $r(A)_{10} \cdot d_{21}$-mer; for n = 50, $r(A)_{50} \cdot d_{21}$-mer. [c] Concentrations of AZTTP: 200, 100, 50, 0 nM. [d] Concentrations of AZTTP: 40, 20, 10, 0 nM. [e] Concentrations of AZTTP: 10, 5, 2.5, 0 nM. [f] Concentrations of AZTTP: 8, 4, 2, 0 nM.

mediately after the primer on template B, all of the 100 [^{3}H]TMP moieties are prevented from being incorporated, and the enzyme has to dissociate from the primer-template in order to catalyze the next reaction. Since the dissociation of HIV-1 RT from primer-template is a very slow process compared to TMP moiety incorporation (Müller et al., 1991; Reardon & Miller, 1990), the experiment with template B would show *less* TMP incorporation (stronger apparent inhibition) than the experiment with template A (weaker apparent inhibition), even under otherwise identical experimental conditions. This hypothesis suggests that the observed inhibitory potency of AZTTP should depend on the number of TMP moieties which can be processively incorporated into a DNA chain: the longer the processive event in a given system, the lower the observed K_i value for AZTTP; the larger the number of AMP moieties in a template, the lower the observed K_i value for AZTTP.

Inhibition of HIV-1 RT by AZTTP with Different RNAs as Templates. In order to test our hypothesis, we created a special set of RNAs as templates to study the competitive inhibition HIV-1 RT by AZTTP. As shown in Table I, the only difference among these RNA templates is the number of adenosyl residues following the sequence complementary to the primer. The K_i values for AZTTP for each template are given in Table I. The results are fully consistent with our hypothesis. For the template allowing only one TMP moiety to be incorporated into the DNA strand, the observed K_i value for AZTTP is 132 nM. For the template allowing 50 TMP moieties to be incorporated, the observed K_i value for AZTTP drops to 8 nM. In agreement with previous literature reports (Eriksson et al., 1989), for the template allowing a large number of TMP moieties to be incorporated, i.e., oligo(dT)·poly(rA), we obtained an observed K_i value of 3.6 nM (Table I).

Our results can at least in part account for the wide range of K_i values for AZTTP reported in the literature. In the case with oligo(dT)·poly(rA) as primer-template, the variation of the reported K_i values (2–35 nM) might arise from the processivity which should depend on assay conditions. Majumdar et al. (1988) reported that the polymerization catalyzed by HIV-1 RT has a high tendency to stop after incorporation of the first NMP moiety. This factor will strongly influence the observed K_i value if the assay conditions interfere with this tendency. From this consideration, the K_i values obtained with a template allowing only one TMP moiety incorporation should be the upper limit and should be constant.

Our finding also infers that *any* HIV-1 RT inhibitor which interferes with the processivity of the enzyme will show the same template-length dependency. That is, the observed K_i value of such an inhibitor would be very different when homopolynucleotides and sequence-defined polynucleotides are used as templates. This effect has two consequences: (1) when one tests an HIV-1 RT inhibitor in experiments with two different RNAs as templates, the K_i values obtained will suggest whether the inhibitor interferes with the processivity; (2) if the inhibitor affects the processivity of the enzyme, one has to decide which K_i value is more meaningful in evaluating the potency of the inhibitor.

Conclusions

Our analysis suggests that AZTTP is not so potent a competitive inhibitor as shown by the K_i values from the experiments using oligo(dT)·poly(rA) as a primer-template. The lower observed K_i values (2–35 nM) are artifacts of the use of homopolynucleotides as templates. This finding is important for the process of discovery of new drugs for the treatment of HIV infection. A common method of evaluating a new drug candidate is to compare it with existing drugs. AZT is the first drug that has FDA approval for the treatment of HIV infection, and if we use the lower, artifactual K_i values for AZTTP as criteria to search for new drugs, we will lose interest in further investigation of some drug candidates simply because of the "large K_i value difference" from AZTTP. We conclude that homopolynucleotides should not be used as a template to study chain-termination inhibitors since the inhibition parameter obtained is less meaningful and cannot be usefully compared with other classes of inhibitors. Rather, we prefer to use an RNA template of defined sequence which allows only one NMP moiety incorporation.

Our results, along with previous reports (Müller et al., 1991; Reardon & Miller, 1990), suggest that 3′-azido substitution does not increase the enzyme-binding affinities of AZTTP and the TMP-terminated primer-template. In other words, neither AZTTP nor its termination products bind to the HIV-1 RT active site better than TTP and the normal primer-template, and AZTTP exerts its effect by DNA chain termination (Müller et al., 1991). From this point of view, two factors should be emphasized for the development of a new chain terminator: one should strive for both better binding to the active site of HIV-1 RT (lower K_m and K_d) and faster turnover rates (higher k_{cat}).

The K_i value is a key parameter for other types of inhibitors to reflect their potencies as well as their dissociation from an enzyme. Despite this, however, our results lead to the conclusion that chain-termination inhibitors of HIV-1 RT should not be characterized solely by their K_i values. In addition to its dependence on the length of the template, a K_i value obtained for a chain terminator can reflect a combination of substrate-binding effects, binding effects of the intermediates generated in the course of reaction, and effects of product release. Since AZTTP and dideoxynucleotide analogues mainly work as chain terminators, their observed K_i values should be interpreted with great care. Perhaps K_m (K_d) and k_{cat} values of chain terminators are more useful in vitro parameters for comparisons of their relative potencies.

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Registry No. AZTTP, 92586-35-1; RT, 9068-38-6; poly(rA), 24937-83-5.

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Noncovalent Interactions of Poly(adenosine diphosphate ribose) with Histones†

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ABSTRACT: Covalent linkage of ADP-ribose polymers to proteins is generally considered essential for the posttranslational modification of protein function by poly(ADP-ribosyl)ation. Here we demonstrate an alternative way by which ADP-ribose polymers may modify protein function. Using a highly stringent binding assay in combination with DNA sequencing gels, we found that ADP-ribose polymers bind noncovalently to a specific group of chromatin proteins, i.e., histones H1, H2A, H2B, H3, and H4 and protamine. This binding resisted strong acids, chaotropes, detergents, and high salt concentrations but was readily reversible by DNA. When the interactions of variously sized linear and branched polymer molecules with individual histone species were tested, the hierarchies of binding were branched polymers > long, linear polymers > short, linear polymers and H1 > H2A > H2B = H3 > H4. For histone H1, the target of polymer binding was the carboxy-terminal domain, which is also the domain most effective in inducing higher order structure of chromatin. Thus, noncovalent interactions may be involved in the modification of histone functions in chromatin.

During DNA excision repair of higher eukaryotes, large numbers of protein-bound adenosine diphosphate ribose (ADP-ribose)[1] polymers are processed by the poly(ADP-ribosyl)ation system of chromatin. The reaction cycle begins with postincisional activation of the enzyme poly(ADP-ribose) polymerase (EC 2.4.2.30), automodification of the enzyme, and modification of other DNA-binding proteins with variously sized ADP-ribose polymers. Protein-bound polymers are then rapidly degraded by the consecutive actions of poly(ADP-ribose) glycohydrolase and ADP-ribosyl protein lyase [for reviews, see Ueda and Hayaishi (1985), Ueda (1987), and Althaus and Richter (1987)].

The molecular mechanism whereby poly(ADP-ribosyl)ation primes chromatin proteins for an active role in DNA excision

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[1] Abbreviations: ADP-ribose, adenosine diphosphate ribose; AMP, adenosine 5′-monophosphate; NAD^+, β-nicotinamide adenine dinucleotide; Tris, tris(hydroxymethyl)aminoethane; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography.

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