Dehydroaltenusin was found to be an inhibitor of mammalian DNA polymerase α (pol α) in vitro. Surprisingly, among the polymerases and DNA metabolic enzymes tested, dehydroaltenusin inhibited only mammalian pol α. Dehydroaltenusin did not influence the activities of the other replicative DNA polymerases, such as δ and ε; it also showed no effect even on the pol α activity from another vertebrate (fish) or plant species. The inhibitory effect of dehydroaltenusin on mammalian pol α was dose-dependent, and 50% inhibition was observed at a concentration of 0.5 μM. Dehydroaltenusin-induced inhibition of mammalian pol α activity was competitive with the template-primer and non-competitive with the dNTP substrate. BIAcore analysis demonstrated that dehydroaltenusin bound to the core domain of the largest subunit, p180, of mouse pol α, which has catalytic activity, but did not bind to the smallest subunit or the DNA primase p46 of mouse pol α. These results suggest that the dehydroaltenusin molecule competes with the template-primer molecule on its binding site of the catalytic domain of mammalian pol α, binds to the site, and simultaneously disturbs dNTP substrate incorporation into the template-primer.

Recent investigations have revealed that eukaryotic cells contain at least eight types of DNA polymerase (pol α, β, γ, δ, ε, ζ, η, and θ) (1–4). We screened for natural compounds that selectively inhibit one of the eukaryotic DNA polymerases and found several inhibitors (5–8). Selective inhibitors of DNA polymerases are useful tools and molecular probes to distinguish DNA polymerases and clarify their biological and in vivo functions (9). For example, aphidicolin is a selective inhibitor of both DNA polymerase (pol) α and eukaryotic DNA replicative polymerase, indicating that this polymerase is essential for DNA replication (10). Aphidicolin inhibitor has been very useful for studying the DNA replication system (11); however, there have been no previous reports of inhibitors capable of distinguishing among pol α, δ, and ε.

We found an interesting inhibitor that influenced only the activity of mammalian pol α. The agent, which was determined to be dehydroaltenusin, has been reported to be the inhibitor of myosin light chain kinase (12, 13). Dehydroaltenusin did not affect the activities of replicative DNA polymerases, such as δ and ε, from calf thymus and HeLa cells, respectively; it also showed no effect on pol α activity even from another vertebrate, i.e. fish, although the amino acid sequences of both DNA polymerases are highly homologous. The agent must be able to determine small structural differences between mammalian and fish pol α. Therefore, dehydroaltenusin could be a useful tool with which to study the biochemical functions of pol α and a molecular probe to distinguish the structure of pol α.

EXPERIMENTAL PROCEDURES

Materials—Nucleotides and chemically synthesized template-primers, such as poly(dA) and oligo(dT)12-18, were purchased from Amer sham Pharmacia Biotech (Uppsala, Sweden). [3H]dTTP (43 Ci/mmol) was purchased from PerkinElmer Life Sciences. M13 DNA was purchased from Takara (Tokyo, Japan). All other reagents were of analytical grade and were purchased from Wako Ltd. (Osaka, Japan).

Enzymes—DNA polymerase α was purified from calf thymus by immunoaffinity column chromatography as described previously (14). The amino-terminal (1–329) and the carboxyl-terminal (1280–1465) truncation mutants of the largest subunit of pol, p110, were expressed in Sf9 cells using the baculovirus expression system. A 3.6-kilobase pair EcoRI-KpnI fragment containing cDNAs of p110 with His6 tag and T7 tag (15) was introduced into EcoRI-KpnI-digested pFastBac1 (CLONTECH). A recombinant virus was generated according to the manufacturer's instructions. The baculovirus-infected insect cells were suspended in ice-cold buffer containing 20 mM sodium phosphate, pH 7.5, 2 mM MgCl2, 10% glycerol, 1 mM dithiothreitol, 0.3 mM KCl, 0.1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM ECTA, 0.4 mg/ml antipain, 0.4 mg/ml aprotinin, 0.1 mg/ml leupeptin, and 80 ng/ml pepstatin A, incubated on ice for 30 min, and then centrifuged at 12,000 × g for 30 min. The supernatant was mixed with co- lant-chelating Sepharose (TALON, CLONTECH) for 1 h. After washing with the buffer containing 50 mM sodium phosphate, pH 7.0, 10% glycerol, 0.3 mM KCl, and 0.1% Nonidet P-40, proteins were eluted with elution buffer containing 50 mM sodium phosphate, pH 5.3, 200 mM imidazole, 10% glycerol, 0.3 mM KCl, and 0.1% Nonidet P-40. The proteins were fractionated by 15–35% glycerol gradient centrifugation as described by Mizuno et al. (15). The smallest subunit of pol α, p46, was purified as described previously (16). Recombinant rat pol β was purified from Escherichia coli JM103 as described by Date et al. (17). Pol δ was purified from calf thymus as described previously (18). Pol ε was purified from HeLa cells as described previously (19). Fish pol α and δ were purified from the testis of cherry salmon (Oncorhyncus masou) (20). Pol ι (ε-like) and pol II (β-like) from a higher plant, i.e. cauliflower, were purified according to the methods outlined by Sakaguchi et al. (21).
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Human immunodeficiency virus (HIV) type-1 reverse transcriptase (recombinant) and the Klenow fragment of pol I from E. coli were purchased from Worthington Biochemical Corp. (Freehold, NJ). T4 DNA polymerase, Taq DNA polymerase, T7 RNA polymerase, and T4 poly- nucleotid kinase were purchased from Takara (Kyoto, Japan). Calf thymus terminal transferase and bovine pancreas deoxyribonuclease I were purchased from Stratagene Cloning Systems (La Jolla, CA). Purified human placenta DNA topoisomerases I (2 units/ml) and IIa (2 units/ml) were purchased from Topogen, Inc. (Columbus, OH).

DNA Polymerase Assays—Activities of DNA polymerases were measured by the methods described previously (5, 6). For DNA polymerases, poly(dA)poly(dT)12–18 and dTTP were used as the template-primer DNA and nucleotide substrate, respectively. For HIV reverse transcriptase, poly(A)poly(dT)12–18 and dTTP were used as the template-primer and nucleotide substrate, respectively. For calf terminal transcriptase, oligo(dT)12–18 (3′-OH) and dTTP were used as template-primer and nucleotide substrate, respectively.

Dehydroaltenusin was dissolved in dimethyl sulfoxide (Me2SO) at various concentrations and sonicated for 30 s. Aliquots of 4 μl of sonicated samples were mixed with 16 μl of each enzyme (final 0.05 unit) in 50 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol, 50% glycerol, and 0.1 mM EDTA and kept at 0 °C for 10 min. These inhibitor–enzyme mixtures (8 μl) were added to 16 μl of each of the enzyme standard reaction mixtures, and incubation was carried out at 37 °C for 60 min with the exception of Taq DNA polymerase, which was incubated at 74 °C for 60 min. The activity without the inhibitor was considered 100%, and the remaining activities at each concentration of the inhibitor were determined as percentages of this value. One unit of each DNA polymerase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of dTTP into synthetic template-primer (i.e. poly(dA)poly(dT)12–18 5′A′T′ = 2′A′) in 60 min at 37 °C under normal reaction conditions for each enzyme (5, 6).

Other Enzyme Assays—Activities of DNA primase, T7 RNA polymerase, T4 poly- nucleotide kinase, and bovine pancreas deoxyribonuclease I were measured in each of the standard assays according to the manufacturer’s specifications as described by Kozumi et al. (22), Nakayama and Saneyoshi (23), Soltis and Uhlenbeck (24), and Lu and Sakaguchi (25), respectively. Telomerase activity was determined using the polymerase chain reaction-based telomeric repeat amplification protocol as described (26) with some modifications (27).

Gel Mobility Shift Assay—The gel mobility shift assay was carried out as described by Casas-Finet et al. (28). The binding mixture (a final volume of 20 μl) contained 20 mM Tris-HCl, pH 7.5, 40 mM KCl, 50 μM bovine serum albumin, 10% Me2SO, 2 mM EDTA, M13 plasmid DNA (2.2 nmol; nucleotide, single-stranded, and singly primed), and 25 pmol of the core domain of the large subunit of pol α. Various concentrations of dehydroaltenusin were added to the binding mixture followed by incubation at 25 °C for 30 min. Samples were run on a 1.2% agarose gel in 0.1 M Tris acetate buffer, pH 8.3, containing 5 mM EDTA at 50 V for 2 h.

Surface Plasmon Resonance—Mammalian pol α and dehydroaltenusin binding analysis was performed using a Biosensor BIAcore instrument, BIAcore X (BIAcore, Sweden). CM5 research grade sensor chips (BIAcore, Sweden) were used. All buffers were filtered before use. The core domain of the largest subunit of mouse pol α, p110, and the smallest subunit of mouse pol α, p46 (314 or 131 μM/ml, respectively; 35 μl each, i.e. 0.1 nmol each), in coupling buffer (10 mM sodium acetate, pH 5.0) was injected over a CM5 sensor chip at 20 μl/min to capture the protein to the carboxymethyl dextran matrix of the chip by N-hydroxysuccinimidyl-N-ethyl-N′-[3′-dimethylaminopropy]carbodiimide hydrochloride (NHS/EDC) coupling reaction (60 μl of mixture) as described (29). Unreacted N-hydroxysuccinimide ester groups were inactivated using 1 M ethanolamine-HCl, pH 8.0. This reaction immobilized about 5000 and 2400 response units of p110 and p46 proteins, respectively. Binding analysis of dehydroaltenusin was performed in running buffer including dehydroaltenusin (5 mM potassium phosphate buffer, pH 7.0, and 10% Me2SO) at a flow rate of 20 μl/min at 25 °C. Kinetic parameters were determined using the software BIAevaluation 3.1.

RESULTS AND DISCUSSION

Production and Isolation of Dehydroaltenusin—We screened for DNA polymerase inhibitors and found a natural compound that inhibits mammalian DNA pol α activity but not pol β activity from a fungus (strain 86H02B04-1(2)) collected from fields in the vicinity of Noda City in Chiba prefecture, Japan. The compounds were extracted with CH2Cl2 from the broth of the fungus and then purified by silica gel column and Sephadex LH-20 column chromatography. Electron impact mass, negative fast atom bombardment high resolution mass, 1H NMR, 13C NMR, and distortionless enhancement by polarization transfer spectroscopic analyses suggested that the inhibitor fraction was dehydroaltenusin, previously reported as an in-
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Effects of Dehydroaltenusin on the Activities of Mammalian DNA Pol α and β and on Other Enzymes—Fig. 2 shows the inhibition dose-response curves of dehydroaltenusin against calf pol α and rat pol β. The inhibition by dehydroaltenusin was dose-dependent. This compound was effective at inhibiting pol α with 50% inhibition observed at a dose of 0.68 μM and with almost complete inhibition at 4 μM (Fig. 2). Because aphidicolin, a potent inhibitor of mammalian pol α, shows complete inhibition at 40 μM (30), the effect of dehydroaltenusin on this enzyme was almost 10-fold stronger than the effect of aphidicolin.

The inhibitory effects of dehydroaltenusin on calf pol α and mouse pol α were more than 100-fold stronger than the inhibitory effects on rat pol β, calf pol δ, and human pol ε, and these effects were approximately 130-fold stronger than the inhibitory effects on human telomerase, HIV type-1 reverse transcriptase, and T7 RNA polymerase (Table I). This compound had no inhibitory effect on pol α or pol δ from the fish (cherry salmon) or higher plant, such as cauliflower pol I (α-like) or pol II (β-like), prokaryotic DNA polymerases, such as the Klenow fragment of E. coli DNA polymerase I, Taq DNA polymerase and T4 DNA polymerase, and other DNA-metabolic enzymes, such as calf terminal transferase and bovine pancreas deoxynucleotidase (Table I). The IC₅₀ values in Table I did not change when the template-primer was activated DNA. Dehydroaltenusin thus appeared to be a selective inhibitor of mammalian pol α.

Mode of DNA Pol α Inhibition by Dehydroaltenusin—Next, to elucidate the mechanism of inhibition, the extent of inhibition as a function of DNA template-primer or dNTP substrate concentration was studied (Fig. 3). In kinetic analysis, poly(dA):oligo(dT)₁₂₋₁₈ and dTTP were used as the template-primer DNA and dNTP substrate, respectively. Double reciprocal plots of the results showed that the dehydroaltenusin-induced inhibition of calf pol α activity was competitive with the DNA template and non-competitive with the dNTP substrate (Fig. 3, A and B). In the case of the DNA template, the apparent maximum velocity (Vₘₐₓ) was unchanged at 55.6 pmol/h, whereas the 140, 220, and 769% increases in Michaelis constant (Kₘ) were observed in the presence of 0.25, 0.5, and 1 μM dehydroaltenusin, respectively (Fig. 3A). The Kₘ for the dNTP substrate was 1.65 μM, and the Vₘₐₓ for the dNTP substrate decreased from 29.2 to 5.36 pmol/h in the presence of 1 μM dehydroaltenusin (Fig. 3B). The inhibition constant (Kᵢ) value, obtained from Dixon plots, was found to be 0.23 and 0.18 μM for the DNA template and substrate dTTP, respectively (Fig. 3, C and D). When activated DNA and four dNTP substrates were used as the template-primer DNA and dNTP substrate, respectively, the inhibition of calf pol α by dehydroaltenusin was competitive with the DNA template and non-competitive with the dNTP substrate (data not shown). The core domain of the largest subunit, p110, of mouse pol α, which has the catalytic activity, was similarly inhibited (data not shown). Biochemically, this mode of action is unusual.

On the other hand, the inhibition of pol α by aphidicolin was non-competitive with activated DNA as a DNA template-primer and competitive with respect to the dNTP substrate (30). Moreover, aphidicolin inhibited pol α by competing with dCTP but not by competing with the other three deoxynucleoside triphosphates (30). In contrast, inhibition of pol α by dehydroaltenusin was non-competitive with the four deoxynucleoside triphosphates (data not shown). The mode of the inhibitory effect of dehydroaltenusin on pol α was quite different from the mode of the inhibitory effect of aphidicolin.

These results suggest that dehydroaltenusin directly binds to the template-primer DNA-binding site of pol α, whereas dehydroaltenusin may bind or interact with a site that is distinct from the dNTP substrate-binding site. Both the DNA-binding site and the dNTP substrate-binding site of pol α occurred in the largest subunit, p110 (31–33). We further studied the interaction between dehydroaltenusin and the largest subunit of pol α.

Analysis of the Binding between Dehydroaltenusin and the Largest Subunit of Mammalian DNA Polymerase α—Mammalian pol α is made up of four subunits, i.e., p180, p68, p54, and p46 (31, 34, 35). The largest subunit, p180, and the smallest subunit, p46, have the catalytic DNA polymerase and DNA primase activities, respectively (31–33). The other subunits, p68 and p54, have no known enzyme activities. We constructed the core domain (p110) in which we deleted the amino-terminal amino acids and applied X-ray crystallography to dehydroaltenusin. The X-ray crystallography data suggested that the amino-terminal amino acids are essential for the inhibition of pol α.

Fig. 3. Kinetic analysis of the inhibition of calf thymus DNA polymerase α by dehydroaltenusin. A, calf pol α activity was measured in the absence (□) or presence of 0.25 (●), 0.5 μM (▲) or 1 μM (●) dehydroaltenusin using the indicated concentrations of the template-primer DNA. B, calf pol α activity was assayed with the indicated concentrations of the substrate dTTP in the presence of 0.25 μM (●), 0.5 μM (▲), or 1 μM (●) or in the absence (□) of dehydroaltenusin. C and D, the inhibition constants (Kᵢ) were determined as 0.23 and 0.18 μM from a Dixon plot made on the basis of the same data for A and B, respectively. Amount of calf pol α in the assay mixture was 0.05 unit.
and the carboxyl-terminal (1280–1465) regions of the largest (p180) and smallest (p46) subunits of pol α, and then the recombinant proteins were expressed and purified (15). We investigated the interaction between the core domain of pol α and dehydroaltenusin. The template-primer DNA-binding protein activity of p110 was analyzed by gel mobility shift assay. Fig. 4 shows the results of gel mobility shift assay of the M13 single-stranded DNA (ssDNA)-110-kDa core domain of the largest subunit of pol α-binding complex. The M13 DNA used was often separated into a major band and a faint band. The nature of the faint bands is currently unclear. These bands may be self-primed linear DNA or, less possibly, circular dimers. Pol α bound to M13 ssDNA and was shifted in the gel (lanes 6 and 9). In the binding assay, M13 ssDNA at 2.2 nmol of nucleotide was added with 25 pmol of the enzyme (lanes 2–6 and lanes 8 and 9). The molecular ratios of dehydroaltenusin (lanes 1–6) or aphidicolin (lanes 7–9) and the enzyme are shown as the inhibitor-to-enzyme ratio (I/E) in Fig. 4. When the I/E ratio was 1 or more, dehydroaltenusin interfered with the complex formation between M13 ssDNA and pol α (lanes 2–4). At a ratio of 0.5, dehydroaltenusin disappeared, suggesting that one molecule of dehydroaltenusin competes with one molecule of M13 DNA and subsequently interferes with the binding of DNA to the largest subunit of pol α. Kinetic analysis indicated that dehydroaltenusin acted by competing with the DNA template on pol α; thus, dehydroaltenusin directly binds the DNA binding site of the largest subunit of pol α and may indirectly inhibit dNTP incorporation by pol α (Fig. 3). On the other hand, the interference of the shift in gel mobility by aphidicolin did not occur (Fig. 4, lane 8), indicating that the modes of action of aphidicolin and dehydroaltenusin on pol α differed from each other. The aphidicolin data were in agreement with the data of a previous study indicating that the mode of inhibition of pol α by aphidicolin was uncompetitive with DNA template-primer (30).

Binding between Dehydroaltenusin and the Subunits of Mammalian DNA Polymerase α—To confirm the kinetic parameters and results of biochemical experiments precisely, the parameters for the binding of dehydroaltenusin were determined using the core domain of the largest subunit of mammalian pol α, p110, and the smallest subunit of pol α, p46, immobilized to the sensor chip in a BIAcore instrument. Five different concentrations of dehydroaltenusin (5, 10, 15, 20, and 25 μM) were injected over 110- and 46-kDa proteins for 2 min at 20 μl/min and dissociated for 3 min at 20 μl/min. The background resulting from injection of running buffer alone was subtracted from the data before plotting.
jugated proteins. Dehydroaltenusin bound to the p110 subunit, which contains the DNA binding and catalytic activity of mammalian pol α and dissociated from the protein (Fig. 5A). The dissociation constant ($K_D$) of the binding of dehydroaltenusin to the p110 subunit was determined to be 0.50 μM (Fig. 5A). On the other hand, dehydroaltenusin hardly bound to the p46 subunit, which has DNA primase activity (Fig. 5B). These results suggest that dehydroaltenusin interacted directly with the catalytic subunit of pol α.

Dehydroaltenusin did not influence the activities of mammalian pol δ and ε, which are the other replicative DNA polymerases; it also showed no effect on pol α of another vertebrate, the cherry salmon. Dehydroaltenusin is a type of antibiotic produced by a fungus and is chemically stable under in vivo conditions, indicating that it may be useful for analyzing the replication system within cells and for clinical use. Aphidicolin, a compound-binding sites.

As described above, dehydroaltenusin is a selective inhibitor of mammalian pol α, and it is known that the compound is also an inhibitor of myosin light chain kinase (12, 13). Biochemical properties of pol α and myosin light chain kinase are totally different from each other. Therefore, why the inhibitor of myosin light chain kinase can inhibit the pol α activity is presently unknown. The solution may lie in the structural analysis of the compound-binding sites.

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