An Unusual Polyanion from *Physarum polycephalum* That Inhibits Homologous DNA Polymerase α in Vitro†

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Received November 17, 1988; Revised Manuscript Received March 9, 1989

ABSTRACT: From extracts of microplasmodia of *Physarum polycephalum* and their culture medium, an unusual substance was isolated which inhibited homologous DNA polymerase α of this slime mold but not β-like DNA polymerase and not heterologous DNA polymerases. Analysis, especially NMR spectroscopy, revealed the major component to be an anionic polyester of L-malic acid and the inhibition to be due to poly(L-malate) in binding reversibly to DNA polymerase α. The mode of inhibition is competitive with substrate DNA and follows an inhibition constant K_i = 10 ng/mL. Inhibition is reversed in the presence of spermine, spermidine, poly(ethylene imine), and calf thymus histone H1. According to its ester nature, the inhibitor is slightly labile at neutral and instable at acid and alkaline conditions. Its largest size corresponds to a molecular mass of 40–50 kDa, but the bulk of the material after purification has lower molecular masses. The inhibitory activity depends on the polymer size and has a minimal size requirement.

In previous studies, Brewer and Rusch (1966) and Schiebel and Schneck (1974) observed a severalfold activation of DNA synthesis in isolated nuclei of *Physarum polycephalum* in the presence of spermine and spermidine. During purification of DNA polymerase, a substantial increase in activity was observed after *Physarum* extracts were passed over DEAE ion-exchange columns (Baer & Schiebel, 1978; Holler et al., 1987).

We describe an unusual polyanion which inhibits homologous DNA polymerase α of *Physarum polycephalum*. It has been isolated from plasmodial extracts and from the culture medium of that organism. The inhibition is counteracted by polyamines.

Materials and Methods

Materials. Plasmodia of *Physarum polycephalum* (strain M_3c VIII, a gift of Dr. Braun, Bern) were grown in shaking culture on semidefined medium at 27 °C in the dark (Daniel & Baldwin, 1964).

DNA polymerase α from *Physarum* was purified according to Weber et al. (1988). Highly purified preparations had a specific activity of 2.5 × 10^4 units/mg. DNA polymerase β-like from *Physarum* was prepared according to Holler et al. (1987) and had a specific activity of 3.3 × 10^3 units/mg. DNA polymerase α from calf thymus (specific activity 4.7 × 10^3 units/mg; Grosse & Kraus, 1981) was a gift of Dr. Grosse (Göttingen). DNA polymerase α from human placenta was a gift of Dr. O. Lavrik (Novosibirsk) (specific activity 8.5 × 10^3 units/mg; Nevisnky et al., 1986), recombinant human DNA polymerase β was a gift of Dr. Wilson (Abbotts et al., 1988), DNA polymerase I from *Escherichia coli* (specific activity 9.7 × 10^3 units/mg; Jovin et al., 1969) was a gift of O. Muise (Regensburg), and an aphidicolin-sensitive DNA polymerase from *Methanococcus vannielii* (specific activity 3.3 × 10^3 units/mg; Zabel et al., 1985) was a gift of Dr. Zabel (Regensburg); highly purified RNA polymerase from *Escherichia coli* and RNA polymerase from *Methanococcus thermolithotrophicus* were gifts of Dr. Thomm (Regensburg) (Thomm & Stetter, 1985).

Deoxyadenosine 5′-[α-^{32}P]triphosphate (400–700 Ci/mmol) was obtained from Amersham-Buchler. DEAE-cellulose was from Whatman, and Sephacryl S300 was from Pharmacia. Bacto-tryptone and yeast extract were from Difco, and hemin chloride was from Fluka. Aphidicolin was from ICI, putrescine was from Calbiochem, spermidine, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA),[1] and pepstatin A were from Sigma, and spermine, poly(ethylene imine), dextrans, and $PhMeSO_2F$ were from Serva. Poly(ethenesulfonic acid) was from Aldrich, and all other chemicals were from Merck.

Chemically synthesized type β poly(L-malate) was a gift of Dr. Vert (Rouen) (Vert & Lenz, 1979).

Purification of the Inhibitor. (A) From Culture Medium. The supernatant of a 3-day-old shaking culture was stirred into DEAE-cellulose (20 g/L medium). The loaded DEAE-cellulose was extensively washed with 0.3 M KCl in buffer A (10 mM potassium citrate, pH 4.6). Retained inhibitor was eluted with 0.7 M KCl in buffer A. After dilution with buffer A to a final concentration of 0.2 M KCl, active fractions again were loaded on DEAE-cellulose, and inhibitor was eluted in a 0.2–1.0 M KCl gradient in buffer A. The activity-containing pool was concentrated by lyophilization and precipitation in 70% ethanol and chromatographed on a Sephacryl S300 column (75 × 1 cm, elution with 0.1 M KCl in buffer A). Further purification was achieved by HPLC on ion exchanger (Bio-Gel TSK DEAE 5PW, 150 × 21.5 mm, elution with a 0.2–0.8 M KCl gradient in buffer A), occasionally by size fractionation on HPLC Bio-Sil TSK 250 (0.79 × 60 cm), and by repeated precipitation with 70% ethanol. The resulting material was dried in vacuo and subjected to analysis. Part of the substance was incubated in 6 N HCl at 110 °C for 18 h and is referred to as "hydrolyzed" inhibitor.

(B) From Plasmodial Extract. Plasmodia (500 g) of the culture above were collected on a cotton sieve as in Holler et al. (1987) and washed with 3 volumes of a solution containing 10 mM glucose and 1 mM calcium phosphate (pH 7). They were homogenized in 2 volumes of buffer A/0.1 M NaCl in a Braun mixer at medium speed for 30 min at room temperature. Protein was denatured and precipitated by stirring 10 min at 55 °C and pelleted after cooling during 1-h cen-

† Supported by the Deutsche Forschungsgemeinschaft.
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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $PhMeSO_2F$, phenylmethanesulfonyl fluoride.

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trifugation at 6000*g* and 4 °C. The inhibitor was purified further as described for the culture medium.

The low-pressure-mixing HPLC system (LKB) consisted of a gradient-forming controller with a mixing valve, a dual-piston HPLC pump (1 mL/min), a He solvent conditioner, and a variable-wavelength UV/vis monitor with a 10-μL/1-cm path cell.

Analysis of the Inhibitor. Protein was determined according to Bradford (1976) with Coomassie Brilliant Blue G250. Amino acids were identified in the hydrolyzed material either after reaction with ninhydrin (Moore & Stein, 1954) or by HPLC on octadecyl Si100 polyol (Serva) after fluorescence labeling with *o*-phthalaldehyde (Larsen & West, 1981).

Neutral sugar was determined photometrically at 620 nm after reaction with anthrone (Morris, 1948; Hollander-Koehler, 1952), amino sugar with *p*-(dimethylamino)benzaldehyde at 585 nm (Strominger et al., 1959), and uronic acid with *o*-hydroxydiphenyl at 520 nm (Blumenkrantz & Asboe-Hansen, 1973).

Phosphate was established according to Eibl and Lands (1969) with ammonium molybdate, sulfate according to Dodgson and Price (1962) with barium chloride, and sulfonic acid according to Abott (1962) with methylene blue. Carboxylic acid was determined according to Kakáč and Vejdelek (1974) by reaction with methanol in the presence of *N*,*N*′-dicyclohexylcarbodiimide and subsequent transfer to the corresponding hydroxamate, which was assayed in the presence of iron(III) chloride. Malic acid was used for calibration. For determination of carboxylic acid ester, the reaction with methanol was omitted (Kakáč & Vejdelek, 1977).

Infrared spectra of native and hydrolyzed inhibitor were recorded in solid KBr (Acculab 7 IR spectrometer, Beckman). ^{1}H NMR spectra of the inhibitor (40 mg/mL in D_2O) and its hydrolysate (40 mg/mL in trifluoroacetic acid) were recorded on an EM NMR 360L spectrometer (Varian). The ^{13}C NMR spectrum of the inhibitor (100 mg/mL in D_2O) was recorded on a WH90 NMR spectrometer (Bruker). The optical rotation of the inhibitor (6 mg/mL in H_2O) was determined in a Perkin-Elmer 241MC polarimeter.

Molecular masses were measured by partition chromatography on Sephacryl S200 or Sephacryl S300 columns (2.5 × 75 cm) with ferritin (440 kDa), catalase (240 kDa), and bovine serum albumin (68 kDa) or with dextrans as standards, and on Bio-Sil TSK 250 (0.75 × 60 cm) or LKB-UltroPac TSK G 4000 SW (0.75 × 60 cm) columns by HPLC (220- or 230-nm detection wavelength) with defined poly(ethylene glycol) and poly(styrenesulfate) (standard kit from Machery Nagel/Düren) as standards. Obtained molecular masses were similar on the basis of the different synthetic, rodlike standards. In comparison, those with proteins as standards were higher by factors of 5–7. Under the assumption of a linear structure of the inhibitor (see below), the former are more realistic. Either 20 mM potassium citrate/0.1 M KCl (pH 4.5) or 10 mM potassium phosphate/0.1 M KCl (pH 7.0) was used as elution buffer.

DNA Polymerase and Inhibitor Assays. The standard DNA polymerase assay was carried out as described for the synthesis of DNA (Holler et al., 1987). [^{3}H]dTTP or [^{32}P]dATP (1 Ci/mmol) was used as the labeled nucleotide. Inhibitor was assayed by activity titration of a fixed amount of *Physarum polycephalum* DNA polymerase α (0.05 unit, phosphocellulose fraction; Weber et al., 1988) in the standard DNA polymerase assay. Always the same preparation of this DNA polymerase α was used. Activity measurements of the other polymerases were performed according to references given under Materials and Methods. Enzyme concentrations were such that (1–3) × 10^4 cpm were measured in the uninhibited controls and that the observed radioactivity was proportional to time and/or enzyme concentration. Specificity and inhibition constants were measured with an inhibitor preparation, which consisted of material 40–50 kDa by more than 50% of its total mass.

The inhibitory material was also assayed by the colorimetric ester reaction modified after Kakáč and Vejdelek (1977). An amount of 160-μL sample was mixed with 10% (w/v) aqueous hydroxylammonium chloride and then with 160 μL of 10% (w/v) aqueous NaOH. After 5 min at room temperature, an amount of 160-μL solution of 4 M HCl and then 160 μL of a 5% (w/v) aqueous solution of $FeCl_3$ were added. The value of the absorbance at 540-nm wavelength (A_{540}) was read after 10 min. The assay is not specific for the inhibitor. The A_{540} value for 50% inhibition of *Physarum* DNA polymerase α was designated "specific activity" [$A_{540}(I = 50)$] [note that increased specific activity is reflected by a lowered $A_{540}(I = 50)$ value].

Since carboxylic acids and esters absorb in the far-UV, the absorbance at 220- or 230-nm wavelength was used for scanning of the HPLC effluent (Scott, 1964).

L-Malate was determined by the method of Bergmeyer (1974) employing mitochondrial malate dehydrogenase (EC 1.1.1.37) from porcine heart (1200 units/mg from Boehringer, Mannheim). The sample (70 μL) was mixed with 850 μL of solution containing 0.4 M hydrazine/0.5 M glycine (pH 9.0) and then with 70 μL of solution of 40 mM NAD. Dehydrogenation of L-malate was started by the addition of 10 μL of malate dehydrogenase suspension (60 units). After 30 min at 37 °C, the amount of NADH was scanned at 340-nm wavelength. Absorbance was standardized against disodium L-malate. For the measurement of poly(L-malate), samples were incubated in the presence of 0.1–0.2 M NaOH for 24 h at 37 °C. After neutralization with 0.1 M HCl, the dehydrogenase assay was performed. The contents of samples were calculated with reference to blinds, which had not been treated with NaOH.

Results

Purification of the Inhibitor. The inhibitor of DNA polymerase α is produced by plasmodia of *Physarum* and reaches maximal concentrations in the culture medium after 3 days of growth. Substantial amounts of protein and nucleic acids were removed from the inhibitor in the plasmodial extract during heat denaturation and precipitation. Strong binding to DEAE-cellulose permits its concentration during the first purification step. Any protein, pigment, slime, and polysaccharide are removed by washing with buffer A containing 0.3 M KCl. Because of increasing viscosity, a Büchner funnel was used in this step. After elution, the inhibitor-containing pool (0.7 M KCl) is almost clear and not viscous. Because of hydrolysis of the inhibitor (see below), the specific inhibitory activity may fall during these steps (see Table I). In late purifications, we used 10 mM potassium phosphate (pH 7.0) instead of citrate buffer in order to minimize hydrolysis; however, the removal of phosphate was problematic at later steps of the purification (dialysis has to be avoided). After the second chromatography on DEAE-cellulose, the inhibitor-containing fraction (0.4–0.7 M KCl) is virtually free of UV-absorbing (260–280 nm) material.

HPLC on DEAE ion exchanger (elution between 0.5 and 0.6 M KCl), precipitation with 70% (v/v) ethanol, and drying over P_2O_5 give a colorless solid of high purity as judged on the basis of the criteria seen below (Table II). It will be

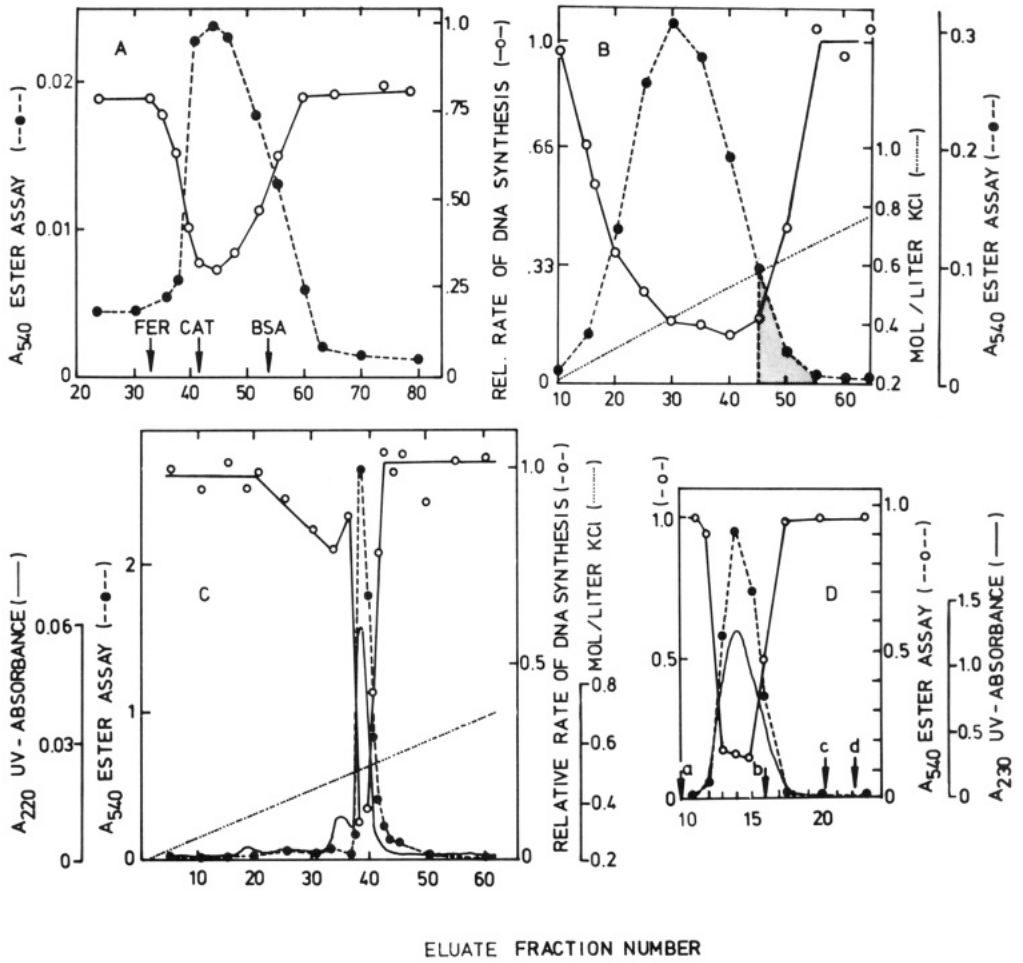

FIGURE 1: Chromatography on DEAE and sieving columns. Contents of eluate fractions were measured as described under Materials and Methods. (Panel A) Medium (10 A_{540} units) from a 3-day-old suspension culture of *Physarum polycephalum* microplasmodia was chromatographed on Sephacryl S200 (4.4-mL fractions collected). Aliquots of 160 μL were analyzed by the ester reaction and 10 μL by the standard DNA polymerase assay. (Panel B) DEAE Bio-Gel A chromatography of 3800 A_{540} units of ester from the second DEAE-cellulose eluate (see Table I). Before the KCl gradient was started, the column was washed with 3 volumes (600 mL) of buffer A/0.2 M KCl (18-mL fractions at 40 mL/h). Aliquots of 50 μL were used in the ester reaction and 10 μL (10-fold diluted) in the standard DNA polymerase assay. (Panel C) Demonstration of high molecular mass material (40–50 kDa according to synthetic markers, see text). The shaded fractions of the eluate in panel B (120 A_{540} units) were loaded on Bio-Gel TSK DEAE 5PW. Washing with 10 mM potassium phosphate buffer (pH 7.0)/0.2 M KCl was followed by HPLC gradient elution (fractions of 4 mL at 1 mL/min). Aliquots of 160 μL were used in the ester reaction and 20 μL (100-fold diluted) in the DNA polymerase assay. (Panel D) Fraction 38 in the preceding chromatography (panel C) was loaded on Bio-Sil TSK 250. Samples (1 mL) were collected by HPLC at a rate of 1 mL/min and assayed in aliquots as in panel C. Molecular mass markers are (a) thyroglobulin (670 kDa), (b) IgG (158 kDa), (c) ovalbumin (44 kDa), and (d) myoglobin (17 kDa).

Table I: Purification Scheme

fraction	A_{540}	$A_{540}(I = 50)$
culture medium (3.5 L)	5000	4.0×10^{-3}
(1) DEAE-cellulose	2500	7.1×10^{-3}
(2) DEAE-cellulose	2000	5.4×10^{-3}
DEAE Bio-Gel A	1800	3.4×10^{-3}
Sephacryl S300	1500	3.5×10^{-3}
DEAE-HPLC	800	2.0×10^{-3}
cell extract (500 g of cells)	8000	17×10^{-3}
55 °C treatment	1600	17×10^{-3}
(1) DEAE-cellulose	1500	5.4×10^{-3}
(2) DEAE-cellulose	1000	4.0×10^{-3}
DEAE Bio-Gel A	700	2.2×10^{-3}

referred to as the potassium salt. One milligram of the material gives rise to 3.4 ± 0.2 A_{540} units.

The results of the purification are summarized in Table I. Values of $A_{540}(I = 50)$ of the inhibitor from the culture medium did not change substantially during purification, while that from the cell extract decreased by a factor of 3–4. After HPLC on Bio-Gel TSK DEAE 5PW, specific activities were the same for preparations from either source. A great part of the observed decrease in yields is due to hydrolysis of inhibitor during purification. It is necessary to accomplish purification within a few days and store the material in the dry form at −60 °C.

The observed properties of both the inhibitor and the purified material were indistinguishable for preparations derived from either plasmodial extracts or culture medium. For the sake of simplicity, we shall make no further distinction between these preparations unless notified.

Inhibitor activity and ester A_{540} may chromatograph in identical positions (Figure 1A,C,D). In many cases, however, the elution profiles are shifted with regard to each other due to hydrolysis, like those in Figure 1B. The degree of "asymmetry" may depend on prolonged standing of preparations at room temperature, exposure to heat, or slightly acidic/alkaline conditions. An extreme case has been investigated by HPLC on DEAE and molecular sieving columns (not shown). More than 70% of the total A_{230}-absorbing material [poly(L-malate)] eluted from the DEAE column at an ionic strength below 0.5 M KCl. Materials of the eluate in extreme low and high positions of the salt gradient were assayed for their molecular masses: 40–50-kDa material in the position 0.55–0.60 M KCl (like in Figure 1B–D) and

Table II: Chemical Group Analysis of the Inhibitor

chemical group	content
protein/peptide	<0.5% of total mass
amino acid	0.2–0.6%[a]
neutral saccharide	<0.5%[b]
amino-substituted saccharide	<0.1%[b]
uronic acid	<0.1%[b]
phosphate	<0.01%[b]
sulfate	<0.2%[b]
sulfonate	<0.1%[b]
carboxylic acid (in hydrolyzed sample)	20 mVal/g[c]

[a] By determination through HPLC, almost every naturally occurring kind of amino acid was identified. [b] Detection limits. [c] Calculated with reference to malic acid (1 mVal = 67 mg) as standard.

4-kDa material between 0.25 and 0.3 M KCl. Specific activities were 6.5×10^{-4} $A_{540}(I = 50)$ and 16×10^{-3} $A_{540}(I = 50)$, respectively. L-Malate (the presumed building block of the inhibitor and the final hydrolysis product) was inactive (maximum concentrations tested were 20 mM). The effects of hydrolysis are shown for controlled saponification of purified poly(L-malate) in Figure 2 [qualitatively, the same results are obtained for synthetic type β poly(L-malate)]. Both the size (molecular mass) and the inhibitory activity decreased as a function of time. In contrast, the ester content (the A_{540} value) decreased by less than 10% of its original value within 30 min (not shown). The overall lifetime of the inhibitory activity was 6 min. Cell extracts and growth media adjusted to neutral pH (4 °C) showed lifetimes of 6–8 days.

The stability of the inhibitor depended on pH. An amount of 5 μg/mL inhibitor was incubated in solutions of different pH at 4 °C for 1 week. Samples of 50 ng were assayed for inhibition of DNA polymerase α. Between pH 4 and 9, the inhibitory activity was not measurably affected under these conditions. In strong acidic and alkaline solutions, the inhibitor was irreversibly inactivated. Under all conditions, the ultimate product was L-malate.

Physical and Chemical Properties. The inhibitor is easily soluble in water and insoluble in organic solvents but becomes soluble in ethanol after hydrolysis in acids. On that basis, lipids and steroids were excluded as major structural components. Nucleic acids and proteins are unlikely because of the absence of absorption bands at 260–280 nm. The strong binding to DEAE ion exchanger at pH 4.6 suggested a net negative charge of the inhibitor.

Table II summarizes results of chemical group analysis. Traces of protein, peptides, and various kinds of amino acids, respectively, were indicated. It was not clarified whether this material was intrinsic to the inhibitor or just an impurity. Of the carboxylic acid, 60% was esterified. After acidic hydrolysis of the inhibitor, all was in the free-acid form. Instead of the blue color, which is routinely expected for the reaction of neutral sugar with anthrone, a red color developed that has been reported in the case of protein, poly(vinyl alcohol) (Hollander-Koehler, 1952), or poly(ethenesulfonate) (result not shown).

According to elementary analysis, a sample of the inhibitor contained by mass 26% carbon, 3% hydrogen, and 19% potassium. Nitrogen, sulfur, and phosphorus were below detection limits.

More specific information was obtained from spectroscopic investigations. The infrared spectrum indicated two different C–H stretching vibrations in positions 3000 and 2940 cm^{-1} as well as C=O stretching vibrations of carboxylic acid or ester, respectively, at 1750–1720 cm^{-1} and of carboxylate ion at 1650–1600 cm^{-1}. The absorption bands that originate from

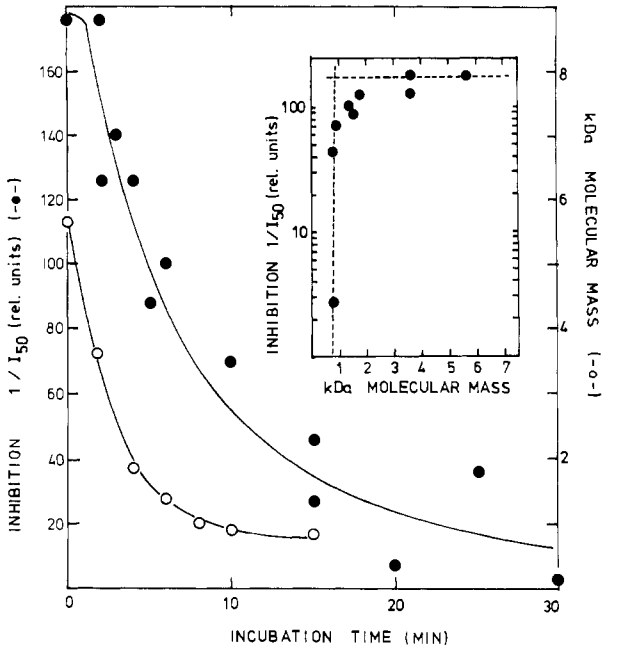

FIGURE 2: Effects of saponification of inhibitor. The reaction mixture (30 °C) contained 1 mg of poly(L-malate), potassium salt, and 0.01 M NaOH. Aliquots of 100 μL were drawn and added to 100 μL of 0.01 M HCl (0 °C) at the times indicated. Of this mixture, 50 μL was spent for molecular mass determination by HPLC, 20 μL for measurement of the inhibition activity (titration of 0.05 unit of *Physarum polycephalum* DNA polymerase α in the standard assay, estimation of the inhibitor concentration, I_{50}, at 50% inhibition), and 100 μL for the measurement of the ester content A_{540}. The minimum size requirement for inhibition by poly(L-malate) is determined from the data plot in the figure inset.

the carboxylate ion are missing in the hydrolyzed material, due to their presence as free acid. In the proton NMR spectrum (Figure 3, panel B), the inhibitor generates two signals: a doublet (chemical shift 2.9 ppm) and a triplet (5.0 ppm). After hydrolysis, the triplet is shifted to 4.6 ppm; the doublet remains unchanged (data not shown). These spectra suggest relatively isolated $-CHCH_2-$ groups in the molecule, with the single proton being located in an electronegative environment that is altered during hydrolysis. The ^{13}C NMR spectrum (Figure 3, panel A) shows four signals derived from the inhibitor. Two signals (36.7 and 72.3 ppm) are indicative of alkyl carbons and two signals (172.3 and 176.1 ppm) of carbonyl carbons. The ^1H as well as the ^{13}C NMR data of the inhibitor and particularly of its hydrolysate are within experimental error consistent with those of malic acid: ^1H NMR, 2.91 and 4.63 ppm (Sadtler standard NMR spectra); ^{13}C NMR, 39.4, 68.0, 172.6, and 175.2 ppm in dioxane (Kalinowski et al., 1984). Furthermore, the specific optical rotation of the inhibitor, $[\alpha]^2_\beta = -13.0 \pm 1.0$ (in units of degrees per gram per decimeter), is between that of L-malic acid ($[\alpha]^2_\beta = -28.6$) and its sodium salt ($[\alpha]^2_\beta = -7.0$).

Saponification of the ester at 37 °C for 24 h in the presence of 0.1 M NaOH completely destroyed its A_{540} units forming property in the ester assay. After neutralization with HCl, this material gave rise to NAD reduction in the stereospecific reaction catalyzed by malate dehydrogenase. One milligram of the ester potassium salt corresponded to the oxidation of 1.13 ± 0.03 mg of disodium L-malate in the standard reaction. If we assume potassium L-malate (molecular mass 155) as the repetition unit of a linear polyester and take into account the molecular mass of 178 of the standard, an equivalent of 1.15 mg is calculated, which agrees well with the experimental value.

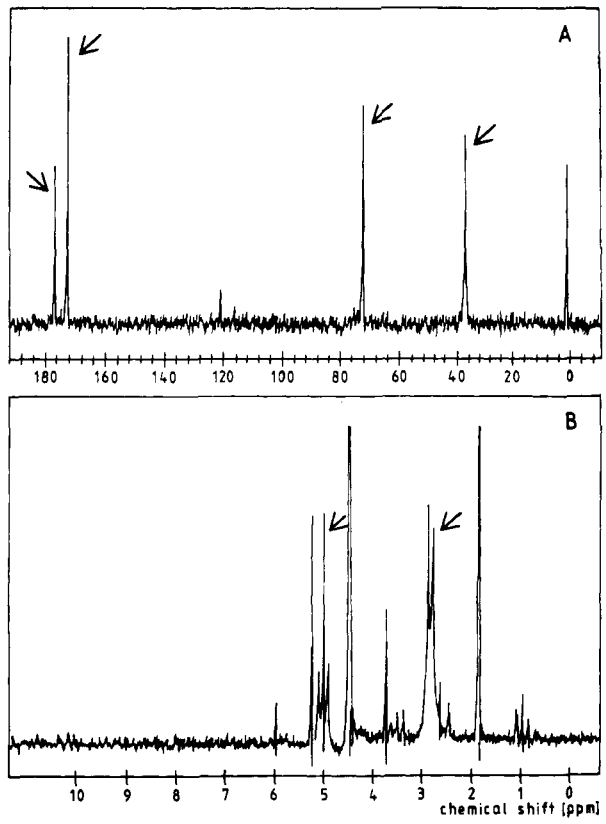

FIGURE 3: NMR spectra of the inhibitor. Signals generated by the inhibitor are indicated by arrows. (A) ^{13}C NMR spectrum: 100 mg/mL in D_2O, signals at 36.7, 72.3, 172.3, and 176.1 ppm, respectively. Acetonitrile (1.6 and 119.9 ppm) was used as standard. (B) ^{1}H NMR spectrum: 40 mg/mL in D_2O, signals at 2.9 (doublet) and 5.0 ppm (triplet). Acetonitrile (1.93 ppm) was used as standard. Additional signals were generated by traces of ethanol (1.0 and 3.4 ppm) derived from the precipitation, and by traces of H_2O in the solvent (4.5 ppm).

The ultraviolet spectrum of the inhibitor exhibited a continuous increase in absorbance toward the far-UV with a shoulder at 205-nm wavelength. Its position was not shifted during saponification, which caused a manifold increase in absorbance, however. In the case of 1.0 mg of ester/mL, the absorbance units (wavelengths) were 0.40 (230 nm), 1.2 (220 nm), 2.65 (210 nm), 4.5 (200 nm), and 10 (190 nm), and for the same solution after saponification and pH neutralization, 8.7 (230 nm), 17 (220 nm), 28 (210 nm), 32 (200 nm), and 100 (190 nm). The UV spectral properties are in accord with malic acid as the building block of the polyester (Scott, 1964).

Structure. On the basis of the presented data, we propose the structure of the inhibitor to be poly(L-malate). The polyester nature is in agreement with our finding that 60% of the carboxylic groups are in the ester form, indicating that most of the "side chain" carboxylic groups were not esterified. Alternative ester linkages in the α- or in the β-positions of the carbon atom that carries the malate hydroxyl group (Figure 4) are possible.

Type β poly(L-malate) had been previously chemically synthesized and characterized (Vert & Lenz, 1979; Braud et al., 1982, 1985; Braud & Vert, 1983; Guerin et al., 1986; Ohuchi, 1985). A sample was obtained from Dr. Vert, Rouen. By all available criteria, synthetic type β poly(L-malate) proved to be identical with the material isolated from *Physarum polycephalum* (see below).

Size and Inhibitory Activity. As mentioned under Purification of the Inhibitor, the inhibitory properties of a sample depended on the size (molecular mass) distribution of its polyester content. Highly purified samples obtained from growth media and cell extracts contained less than 10% of their total ester as material of molecular mass 40–50 kDa. The bulk material had 4–10 kDa. The (apparent) specific inhibitory activity had $A_{540}(I = 50)$ values of (2–4) × 10^{-3}. Samples of synthetic type β poly(L-malate) contained material of molecular mass 30–60 kDa (approximately 50% of their total ester content) and had activity $A_{540}(I = 50)$ values of (2–3) × 10^{-4}.

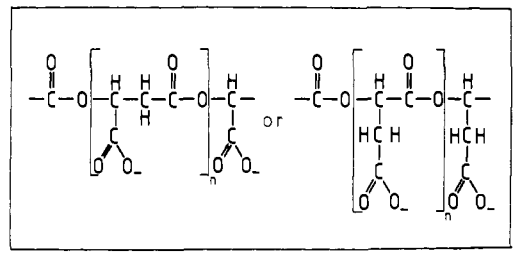

FIGURE 4: Structures of the inhibitor. The inhibitor is proposed to be a polyester of L-malic acid. Two structures are possible: type α poly(L-malate) (right) and type β poly(L-malate) (left).

The data from the hydrolysis experiment in Figure 2 are replotted in the figure inset in terms of the logarithm of $[A_{540}(I = 50)]^{-1}$ versus molecular mass of poly(L-malate). This corresponds to plotting the free energy of inhibitor–polymerase complex formation as a function of inhibitor size. The dependence revealed a sharp drop in affinity below 1000-Da molecular mass referred to a minimal chain length requirement of approximately 10 malate residues in order to achieve inhibition. Because of the size heterogeneity and the variance in molar concentration during the course of hydrolysis, this should be regarded as an approximation.

Specificity of the Inhibitor and of Synthetic Type β Poly(L-malate) for DNA Polymerase α. Purified inhibitor and synthetic type β poly(L-malate) at concentrations of 0.0, 0.02, 0.2, 2, and 20 μg/mL were tested for inhibition of a variety of highly purified polymerases: *Physarum polycephalum* DNA polymerase β-like, human placenta DNA polymerase α, human DNA polymerase β, calf thymus DNA polymerase α, *Escherichia coli* DNA polymerase I (and Klenow fragment) and RNA polymerase, *Methanococcus vannielii* DNA polymerase α-like, and *Methanococcus thermolithotrophicus* RNA polymerase. None was inhibited except calf thymus DNA polymerase α, though 20 μg/mL was required for 50% inhibition in contrast to 0.02 μg/mL for highly purified DNA polymerase α of *Physarum polycephalum.* It was verified that this surprisingly high specificity was not due to specific assay conditions: (i) Inhibition measurements were performed under exactly the same conditions as those for controls [in the absence of poly(L-malate)]. (ii) Variation of pH (pH 7–8) and of concentrations (10-fold) of assay components had no effect on specificity. (iii) A variation in the sequence of addition of reactants and of poly(L-malate) gave identical results. (iv) Results were reproduced by different performing persons. It was also verified that the state of purification of several of these polymerases used in the inhibition assays did not affect inhibition.

Inhibition Is Competitive with DNA. In first experiments, it was verified that inhibition of *Physarum* DNA polymerase α by poly(L-malate) was not irreversible. Thus, inhibition could be almost completely reversed after addition of, for instance, poly(ethylene imine) (Weber et al., 1988) or 0.2 mM spermine (see below), and this activity was aphidicolin-sensitive. Furthermore, inhibition was not inferred by sensitivity of accessory proteins such as DNA primase, because they had been completely removed in homogeneous DNA polymerase

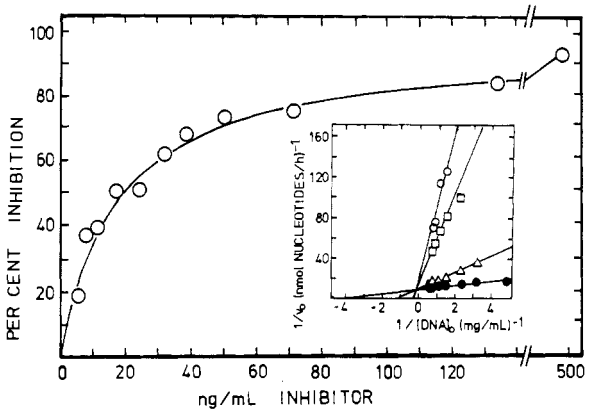

FIGURE 5: Inhibition of *Physarum polycephalum* DNA polymerase α. At a fixed concentration of 15 fmol of highly purified enzyme, the concentration of poly(L-malate) was varied in the standard DNA polymerase assay (main figure). The mode of inhibition and the inhibition constant were measured with increasing concentrations of DNA at various fixed concentrations of poly(L-malate) in the standard assay (figure inset). Initial velocities were plotted according to Lineweaver and Burk (1934). Inhibitor concentrations were 0 (●), 30 (△), 150 (□), and 300 ng/mL (○).

preparations (Weber et al., 1988).

By varying the concentrations of substrate dNTP and DNA, it was further verified that the inhibitor was competitive with DNA (Figure 5) but did not affect K_m values for dNTPs (noncompetitive inhibition, data not shown). Reciprocal plots according to Lineweaver and Burk (1934) (Figure 5, inset) or Eadie (1942) (not shown) were strictly linear, indicating the absence of cooperative reactions. The value of the inhibition constant was K_i = 10 ng/mL. This compares with K_m = 250 μg/mL for activated salmon testis DNA and is indicative of a much tighter binding of poly(L-malate) than of activated DNA to the polymerase. In Figure 5, the activity of 15 fmol (0.1 nM) of highly purified *Physarum* DNA polymerase α was titrated with inhibitor. Concentration at half-maximum inhibition was 20 ng/mL inhibitor.

Inactivation of the Inhibitor by Histone H1 and Polyamines. The activity of DNA polymerase in crude extracts of plasmodia of *Physarum* could be stimulated considerably by addition of polyamines (spermidine, spermine) and poly(ethylene imine) (not shown) to the reaction mixture. The observation was related to the action of purified inhibitor on DNA polymerase α by examining the effect of increasing concentrations of spermine, spermidine, putrescine, and histone H1, respectively (Figure 6). Each of them was found to inactivate the inhibitor. The potency was dependent on the number of amino groups in the polyamine, decreasing in the order spermine > spermidine > putrescine. Histone H1 was by far the most potent effector on a molar basis. The action of 30 ng/mL inhibitor was almost completely reversed in the presence of 2 μM histone H1. Half-maximal "activation" was observed for 0.3 mg/mL poly(ethylene imine) added to plasmodial extracts (Weber et al., 1988). Polyamines themselves did not stimulate DNA synthesis catalyzed by highly purified DNA polymerase from *Physarum polycephalum*. The slight stimulation observed with histone H1 (in the absence of inhibitor) cannot be explained at present.

Discussion

An ester with the structure of poly(L-malate) has been purified from plasmodial extracts and from the culture medium of *Physarum polycephalum*. No difference between preparations from either source has been observed with regard to the analysis presented here. This does not exclude minor structural variations, which might have escaped detection. By the criteria of chromatographic coelution and polyanionic structure, the discovered polymalate is identified as DNA polymerase α inhibitor. The identification is reinforced by the finding that chemically synthesized type β poly(L-malate) has all the characteristic structural and functional properties of the inhibitor and of the isolated material. It also supports the assumption that the inhibitor poly(L-malate) is in the form of the β-ester. Work is in progress in order to confirm this structural aspect by high-resolution NMR spectroscopy.

Poly(L-malate) forms complexes with *Physarum polycephalum* DNA polymerase α of high affinity. The Gibbs free energy of complex formation depends on the size (molecular mass) of the polymer and has a minimum requirement of approximately 10 malate residues. The site of interaction is probably the DNA binding cleft because of reversible inhib-

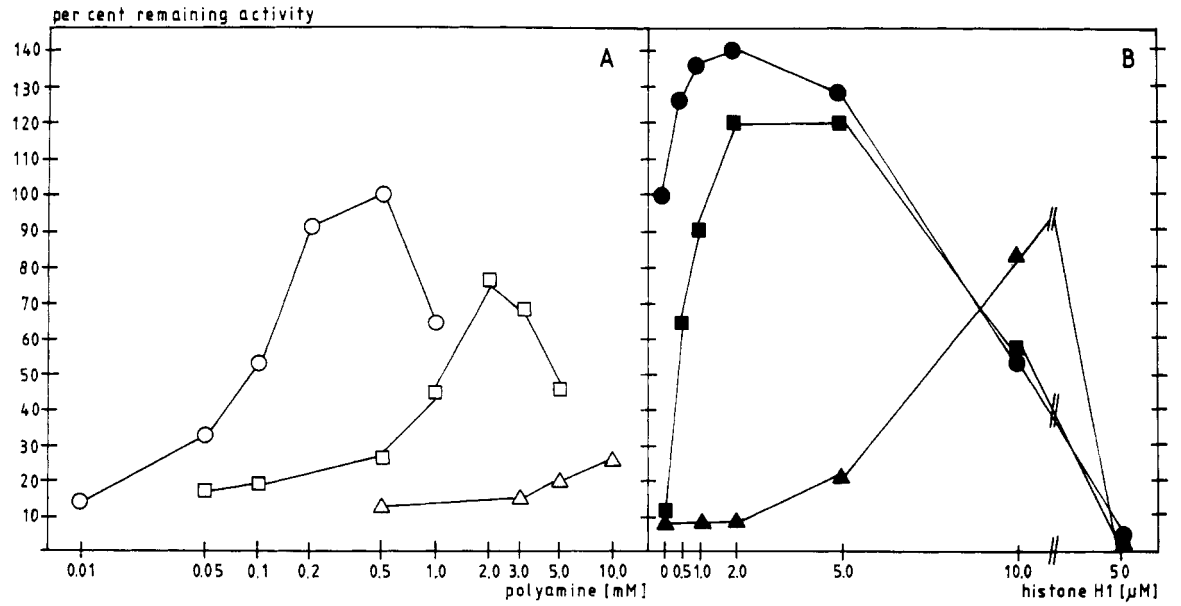

FIGURE 6: Inactivation of the inhibitor by polyamines and by histone H1. (A) Effects of the polyamines spermine (○), spermidine (□), and putrescine (△). Assay mixtures contained 0.05 unit of DNA polymerase α, 30 ng/mL inhibitor, and polyamine in the concentration indicated. (B) Effect of histone H1. Assay mixtures contained 0.05 unit of DNA polymerase α, 0 (●), 30 (■), and 300 ng/mL (▲) inhibitor, respectively, and hiistone H1 in the concentration indicated. Activity of DNA polymerase α in the absence of effector is set at 100%.

ition of DNA synthesis and of competition with substrate DNA. DNA footprinting indicated that the Klenow fragment (of *E. coli* DNA polymerase I), when bound at a primer terminus, covered about 8 bp of duplex DNA upstream of the primer terminus (Joyce et al., 1986) and the efficiency of priming DNA polymerases for DNA synthesis is markedly reducd for oligonucleotides consisting of less than 9–10 nucleotides (Nevinsky et al., submitted for publication). Furthermore, type β poly(L-malate) [but not type α poly(L-malate), which is shorter] can be regarded as roughly an isoster with the ribose 5′-phosphate backbone if about equal distances between the interconnecting atoms between the carboxylate and the phosphate groups are assumed.

The anionic properties of DNA are also reflected by the tendency of poly(L-malate) to bind to polyamines and especially to histone H1 (and probably to any histone).

According to its selective inhibition of only the endogenous DNA polymerase α, poly(L-malate) exhibits a surprisingly high specificity for the type and organismic source of DNA polymerase. The structural requirement for this specificity is not understood at present. It is certainly not the polyanion status of the polymer alone, since a variety of other polyanions tested [see Bernfeld (1963) and Bach (1964)] showed inhibition only in the 10 μg/mL concentration range or higher (results not shown) and are considered unspecific. In an effort to screen for the occurrence of poly(L-malate) among fungi, we have discovered so far one other syncytic organism that contains a type of poly(L-malate) that is a powerful inhibitor of endogenous DNA polymerase (unpublished experiments). However, this one is structurally different, presumably by carrying chemical modifications in the position of the α-carboxylate side chain. Considerable structural work is in progress toward an understanding of the specificity requirements and of structure–function correlations of poly(L-malate) derivatives.

Physarum polycephalum plasmodia are syncytic cells with a high degree of synchronous nuclei division (Holt, 1980). We have measured the concentration and the specific inhibition activity of contained poly(L-malate) under a variety of conditions of the nuclear division cycle and of growth densities by employing the malate dehydrogenase assay and the inhibition assay (unpublished results). We found that (i) poly(L-malate) resided mainly in the nucleus (at concentrations comparable to that of DNA), (ii) its specific inhibition activity cycled betwen nuclei divisions, being low in the S-phase, and (iii) its concentration in the culture medium rose dramatically during growth arrest at the onset of differentiation to spherules. These observations and the occurrence of poly(L-malate) or a derivative thereof in at least one other organism point to one or perhaps several biological functions of poly(L-malate).

The low concentrations required for inhibition, the high specificity, the alternative interactions with polyanions and histones, and the relative ease of (metabolic) convertibility of the polymalate ester bond suggest that this new biopolymer might interact with DNA polymerase α of *Physarum polycephalum* also under in vivo conditions. Our hypothesis is that it functions as a coordinator in DNA synthesis by alternatively interacting with DNA polymerase α (G2-phase) and histones/biogenic amines (S-phase). This may be important for the coordinate synthesis of histones/DNA and their installment into chromatin, at least in syncytic cells. We are engaged in providing experimental evidence in support of the hypothesis and also in the exploration of the biosynthetic pathway of poly(L-malate).

ACKNOWLEDGMENTS

The technical assistance of B. Gantz and H. Reisner is gratefully acknowledged. We are indebted to Dr. Vert, Rouen, for the supply of type β poly(L-malate).

Registry No. Poly(L-malate), 26999-59-7; DNA polymerase, 9012-90-2.

REFERENCES

Abott, D. C. (1962) *Analyst 87*, 286–293.

Abbotts, J., SenGupta, D. N., Zmudzka, B., Widen, S. G., Notario, V., & Wilson, S. H. (1988) *Biochemistry 27*, 901–909.

Bach, M. K. (1964) *Biochim. Biophys. Acta 91*, 619–626.

Baer, A., & Schiebel, W. (1978) *Eur. J. Biochem. 86*, 77–84.

Bergmeyer, H. U. (1974) *Methoden der enzymatischen Analyse*, pp 1632–1639, Verlag Chemie, Weinheim.

Bernfeld, P. (1963) in *Metabolic Inhibitors* (Hochster, R. M., & Quastel, J. H., Eds.) Vol. II, pp 437–472, Academic Press, New York.

Blumenkrantz, N., & Asboe-Hansen, G. (1973) *Anal. Biochem. 54*, 484–489.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Braud, C., & Vert, M. (1983) *ACS Polym. Prep. 24*, 71–72.

Braud, C., Bunel, C., Vert, M., Bouffard, P., Clabaut, M., & Delpech, B. (1982) *Proceedings of the 28th IUPAC International Symposium on Macromolecules at Amherst*, p 384, Amherst, MA.

Braud, C., Bunel, C., & Vert, M. (1985) *Polym. Bull. (Berlin) 13*, 293–299.

Brewer, E. N., & Rusch, H. P. (1966) *Biochem. Biophys. Res. Commun. 25*, 579–584.

Daniel, J. W., & Baldwin, H. H. (1964) *Methods Cell Physiol. 1*, 9–13.

Dodgson, K. S., & Price, R. G. (1962) *Biochem. J. 84*, 106–110.

Eibl, H., & Lands, W. E. M. (1969) *Anal. Biochem. 30*, 51–57.

Grosse, F., & Krauss, G. (1981) *Biochemistry 20*, 5470–5475.

Guerin, P., Francillette, J., Braud, C., & Vert, M. (1986) *Makromol. Chem., Makromol Symp. 6*, 305–314.

Hollander-Koehler, L. (1952) *Anal. Chem. 24*, 1576–1579.

Holler, E., Fischer, H., Weber, C., Stopper, H., Steger, H., & Simek, H. (1987) *Eur. J. Biochem. 163*, 397–405.

Holt, E. C. (1980) in *Growth and Differentiation in Physarum polycephalum* (Dove, W. F., & Rusch, H. P., Eds.) pp 9–63, University Press, Princeton, NJ.

Jovin, T. M., England, P. T., & Bertsch, L. L. (1969) *J. Biol. Chem. 244*, 2996–3008.

Joyce, C. M., Ollis, D. L., Rush, J., Steitz, T. A., Konigsberg, W. H., & Grindley, N. F. F. (1986) *UCLA Symp. Mol. Cell. Biol. 32*, 197–205.

Kakăc, B., & Vejdelek, Z. J. (1974) *Handbuch der Photometrischen Analyse organischer Verbindungen*, Vol. I, Verlag Chemie, Weinheim.

Kakăc, B., & Vejdelek, Z. J. (1977) *Handbuch der photometrischen Analyse organischer Vorbindungen*, Vol. I, Supplment No. 1, Verlag Chemie, Weinheim.

Kalinowski, H.-O., Berger, S., & Braun, S. (1984) *^{13}C-NMR-Spektroskopie*, G. Thieme Verlag, Stuttgart.

Larsen, B. R., & West, G. (1981) *J. Chromatogr. Sci. 17*, 259–265.

Lineweaver, H., & Burk, D. (1934) *J. Am. Chem. Soc. 56*, 658–666.

Moore, S., & Stein, W. H. (1954) *J. Biol. Chem. 211*, 907–913.

Morris, D. L. (1948) *Science 107*, 254–255.

Nevinsky, G. A., Levina, A. S., Podust, V. N., Khalabuda, O. V., & Lavrik, O. I. (1986) *Bioorg. Khim. 12*, 357–368.

Schiebel, W., & Schneck, U. (1974) *Hoppe Seyler's Z. Physiol. Chem. 355*, 1515-1525.
Scott, A. I. (1964) *Interpretation of the ultraviolet spectra of natural products*, p 38, Pergamon Press, Oxford.
Strominger, J. L., Park, J. T., & Thompson, R. E. (1959) *J. Biol. Chem. 234*, 3263-3268.
Thomm, M., & Stetter, K. O. (1985) *Eur. J. Biochem. 149*, 345-351.
Weber, C., Fischer, H., & Holler, E. (1988) *Eur. J. Biochem. 176*, 199-206.
Zabel, H.-P., Fischer, H., Holler, E., & Winter, J. (1985) *Syst. Appl. Microbiol. 6*, 111-118.

Molecular Organization of Developmentally Regulated *Dictyostelium discoideum* Ubiquitin cDNAs†

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Received December 9, 1988; Revised Manuscript Received March 9, 1989

ABSTRACT: *Dictyostelium discoideum* ubiquitin mRNAs are regulated in a complex fashion during spore germination and multicellular development. Species of mRNA of 1900, 1400, 1100, 840, 580, and 500 nucleotides (nt) are found which are expressed differentially during different stages of development. DNA blot analysis indicates that ubiquitin genes constitute a multigene family of at least six genes. cDNAs representing all the ubiquitin mRNA transcripts were isolated and sequenced. The *Dictyostelium* mRNAs are organized as tandem repeats of the 76 amino acid ubiquitin unit (228 nt). We isolated one cDNA containing seven of these tandem repeats, and two different five- and three-repeat cDNAs. In addition, 2 cDNAs containing a single ubiquitin repeat fused at its 3′ end to an unreleated 52 and 78 amino acid extension were identified. There is a remarkable similarity in the sequences of the non-ubiquitin extensions among yeast and mammalian counterparts. The extensions are very basic, containing approximately 30% lysine/arginine. Another common feature of these proteins is the presence of a common structural motif containing cysteine residues at conserved positions, suggesting a metal binding domain that matches a consensus sequence of *Xenopus* transcription factor TFIIIA and other nucleic acid biniding proteins. The characterization of ubiquitin cDNAs and genomic sequences in *D. discoideum* now makes the understanding of its developmental regulation feasible.

The simple eukaryote *Dictyostelium discoideum* has been used as a paradigm to study developmental phenomena. In this organism, the depletion of the food supply sets in motion a schedule of development which results in the orderly succession of developmental events, and which directs when and to what extent in the process, and where in the developing organism, a phenotypic trait will be expressed. Aggregation of individual cells in response to a chemotactic stimulus triggers new cell programs of gene expression that allow the construction of a multicellular structure. Once in the aggregate, the cells are now subject to signals depending on their position within the multicellular structure. These signals dictate types of programs of gene expression that will be followed, e.g., will the cell form a spore, stalk (Sussman & Brackenbury, 1976).

We have been interested in identifying proteins and mRNAs that are exclusively expressed during specific stages of spore germination or multicellular development (Dowbenko & Ennis, 1980; Kelly et al., 1983; Giorda & Ennis, 1987). During our studies, we isolated a cDNA, denoted pLK229, which showed an unusual pattern of regulation during *D. discoideum* development and which coded for a protein identical with human ubiquitin, except that proline in position 19 and threonine in position 22 of the human species were glycine and asparagine, respectively, in *Dictyostelium* (Giorda & Ennis, 1987). An interesting cDNA clone containing a basic polypeptide linked to the C-terminal end of ubiquitin has also been described (Westphal et al., 1986; Müller-Taubenberger et al., 1988a,b).

RNA blot analysis of RNA from vegetative cells, germinating spores, and multicellular development indicates that pLK229-specific mRNA is developmentally regulated (Giorda & Ennis, 1987; Westphal et al., 1986; Müller-Taubenberger et al., 1988a). mRNA species of about 1900, 1400, 1100, 840, 580, and 500 nucleotides (nt) which hybridized to pLK229 plasmid DNA were observed. The 1400-nt species was the only mRNA present in spores. At 1.5 h of spore germination, four mRNAs were observed of 1900, 1400, 1100, and 840 nt, whereas at 3 h germination the predominant species were the 1900- and 1400-nt mRNAs and only traces of the 1100- and 840-nt mRNAs were present. Little of the 1900- to 840-nt mRNAs was present in growing cells, but two new bands at 580 and 500 nt were observed. During multicellular development on filters, these two mRNAs disappeared, and the 1900- and 1400-nt species predominated.

Southern blots of *Dictyostelium* DNA digested with various restriction enzymes demonstrated that the pLK229 genes constituted a multigene family of at least six genes (Giorda & Ennis, 1987; Westphal et al., 1986). As had been described for other organisms, the ubiquitin genes and mRNAs are composed of identical tandem repeats of the 76 amino acid ubiquitin protein (polyubiquitin) (Dworkin-Rastl et al., 1984; Özkaynak et al., 1984; Vierstra et al., 1986; Arribas et al.,

† The nucleic acid sequences in this paper have been submitted to GenBank under Accession Number J02858.
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0006-2960/89/0428-5226$01.50/0