Mechanism of Hemin Inhibition of Erythroid Cytoplasmic DNA Polymerase†

John J. Byrnes,*‡ Kathleen M. Downey, Laura Esserman, and Antero G. So§

ABSTRACT: Hemin, which has an important role in the regulation of hemoglobin synthesis, also regulates the activity of cytoplasmic DNA polymerase from erythroid hyperplastic bone marrow cells and reticulocytes. Hemin inhibits DNA synthesis by binding reversibly to the enzyme. Binding assays demonstrated that hemin prevents association and causes dissociation of the DNA–enzyme complex. This is in contrast to inhibitory compounds that specifically interact with DNA such as ethidium bromide and daunomycin which have little or no effect on the DNA polymerase–template complex. Kinetic analysis reveals that hemin inhibition of DNA synthesis is competitive with respect to template and noncompetitive with respect to substrate. The inhibitory effect of hemin can be reversed by subsequent addition of globin, indicating that the inhibition of DNA synthesis by hemin is not due to irreversible inactivation of the enzyme.

It is now well established that there are two major species of DNA polymerase in eukaryotic tissues (Weissbach *et al.*, 1971; Baril *et al.*, 1971; Sedwick *et al.*, 1972; Chang and Bollum, 1972): a 3–4S DNA polymerase which is the only DNA polymerase found in highly purified nuclei prepared from most tissues, and a large DNA polymerase (8–11.7S depending on ionic strength) found primarily in the cytoplasm (Byrnes *et al.*, 1973; Spadari *et al.*, 1974). That the large DNA polymerase is indeed a cytoplasmic enzyme is strengthened by our recent observation that a substantial amount of large DNA polymerase is present in the rabbit reticulocyte, an anucleate cell (Byrnes *et al.*, 1974a). Although this polymerase has been found in the cytoplasm for some time, the role of this enzyme remains unclear. It has been suggested that this enzyme may be involved in cytoplasmic processing of genetic information (Byrnes *et al.*, 1973, 1974b).

Hemin, the prosthetic group of hemoglobin, plays a crucial role in the regulation of hemoglobin synthesis. The synthesis of hemin is linked to the synthesis of globin. When the concentration of hemin is in relative excess, it inhibits its own synthesis through a feedback mechanism by inhibiting the activity of δ-aminolevulinic acid synthetase (ALA synthetase) (Scholnick *et al.*, 1969; Bottomley and Smithee, 1969). However, hemin stimulates the synthesis of globin and is required for continued initiation of globin synthesis and for maintenance of the polysome profile in reticulocyte extracts (Grayzel *et al.*, 1966; Waxman and Rabinowitz, 1966). We have observed that hemin, at the concentration optimal for globin synthesis, inhibits not only reticulocyte RNA-dependent RNA polymerase activity (Downey *et al.*, 1973), but also the activities of reticulocyte (Byrnes *et al.*, 1974a) and erythroid hyperplastic bone marrow cytoplasmic DNA polymerase (Byrnes *et al.*, 1973).

In continuing our investigation of the role of the cytoplasmic DNA polymerase and its possible regulatory mechanisms, we have investigated the molecular mechanism of hemin inhibition of cytoplasmic DNA polymerase, hoping to elucidate the role of hemin as a modulator of the activities of the cytoplasmic polymerases and the relation of these enzymes to globin synthesis.

Materials and Methods

Tritium-labeled deoxyribonucleoside triphosphates were purchased from New England Nuclear or International Chemical and Nuclear Corporation. Unlabeled deoxyribonucleoside triphosphates were obtained from Sigma Chemical Co. or Calbiochem. Both unlabeled and ^{14}C-labeled poly[d(A-T)] (1–5 × 10^6 daltons) were obtained from General Biochemicals and were dialyzed before use against 0.01 M Tris-HCl buffer (pH 7.4) containing 0.06 M KCl. Calf thymus DNA and DNase were obtained from Worthington Biochemicals. Hemin chloride, ethidium bromide, and daunomycin were obtained from Calbiochem. Activated calf thymus DNA was prepared as described by Aposhian and Kornberg (1962). Bence-Jones protein was a gift of Dr. Duane Schultz, human globin was a gift of Dr. Bruce Cameron, and carbonic anhydrase was a gift of Dr. Philip Whitney.

DNA Polymerase Assay. Cytoplasmic DNA polymerase was prepared from erythroid hyperplastic bone marrow and assayed as previously described (Byrnes *et al.*, 1973). The reaction mixture when poly[d(A-T)] was used as template contained: 40 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)[1] (pH 7.0); 80 mM KCl; 0.4 mM $MnCl_2$; 0.48 mM dATP and 8 μM [^{3}H]TTP, 1000 Ci/mol, or 0.48 mM TTP and 8 μM [^{3}H]dATP, 1000 Ci/mol; 0.24 A_{260} unit/ml of poly[d(A-T)]; and 40 μg/ml of DNA polymerase in a final volume of 0.25 ml. The reaction mixture was incubated for 10 min at 37°.

Binding Assay. The reaction mixture contained in a final

† From the Departments of Medicine and Biochemistry, University of Miami, and the Department of Medicine, Veterans Administration Hospital, Miami, Florida 33152. *Received September 26, 1974.* This research was supported by grants from the National Institutes of Health (NIH AM 09001), the American Heart Association (72-967), and in part by grants from the United Fund and an American Cancer Society Institutional Grant.

‡ Veterans Administration Research Associate.

§ Investigator, Howard Hughes Medical Institute. Portions of this work were done during the tenure of an Established Investigatorship of the American Heart Association.

[1] Abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

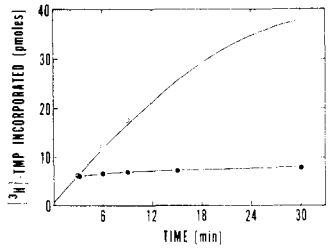

FIGURE 1: Instantaneous inhibition of DNA synthesis by hemin. Assay conditions were as described under Materials and Methods except for the time of incubation. Hemin (1×10^{-5} M) was added after 3 min and aliquots were taken as indicated (●).

volume of 0.25 ml: 40 mM Hepes (pH 7.0); 1.6 mM $MnCl_2$; 2 μg of [^{14}C]poly[d(A-T)], 8000 cpm/μg; and 12–16 μg of DNA polymerase. After incubation at 20° for 4 min the reaction was stopped by rapid chilling and the addition of 2 ml of ice-cold washing buffer containing 0.01 M Tris-HCl (pH 7.8), 0.02 M KCl, and 5.0 mM 2-mercaptoethanol. The reaction mixture was filtered on a Millipore membrane filter (type HA, 2.4-cm diameter), previously soaked in washing buffer, and washed with 60 ml of washing buffer. The filter was dried and counted in a toluene–Omnifluor solution in a liquid scintillation spectrometer.

Gel Filtration of DNA Polymerase and Hemin. A 0.9 × 28 cm column of Bio-Gel A-1.5m was equilibrated with buffer containing: 0.05 M Tris-HCl (pH 7.8), 1.0 mM dithiothreitol, 0.1 mM EDTA, 0.05 M KCl, and 25% glycerol at 4°. Either 330 μg of DNA polymerase, 2.4×10^{-2} μmol of hemin, or a mixture of both in a final volume of 1 ml was applied to the column. Fractions of 1 ml were collected and assayed for DNA polymerase activity as described and for hemin by absorbance at 420 nm. In the absence of hemin the recovery of DNA polymerase activity was 65%.

Gel Filtration of DNA and Hemin. A 0.9 × 28 cm column of Bio-Gel A-1.5m was prepared as described above. Either 700 μg of activated calf thymus DNA, 2.4×10^{-2} μmol of hemin, or a mixture of both in a final volume of 1 ml was applied to the column. Fractions of 1 ml were collected and assayed for DNA by absorbance at 260 nm and for hemin by absorbance at 420 nm.

Results

Effect of Hemin on DNA Synthesis. A time course for synthesis of DNA is shown in Figure 1. In the absence of hemin DNA synthesis continues for at least 30 min. However, when hemin is added after 3 min of incubation, the synthesis of DNA is inhibited instantaneously. The instantaneous inhibition of chain elongation could be due to the interaction of hemin with either the enzyme or the template.

Dissociation of Template–Polymerase Complex by Hemin. The incubation of DNA and DNA polymerase results in the formation of a DNA–enzyme complex which is retained on a cellulose nitrate membrane filter, whereas the enzyme and DNA separately are not retained. This is similar to the retention of the DNA–RNA polymerase complex by cellulose nitrate filters (Jones and Berg, 1966). The effect of increasing concentrations of hemin on the formation of a [^{14}C]poly[d(A-T)]–enzyme complex is shown in Figure 2. Hemin at a concentration of 1.2×10^{-5} M or higher completely inhibits the formation of the [^{14}C]poly[d(A-T)]–enzyme complex and no radioactivity is retained on the Millipore filter. Preincubation of DNA polymerase with

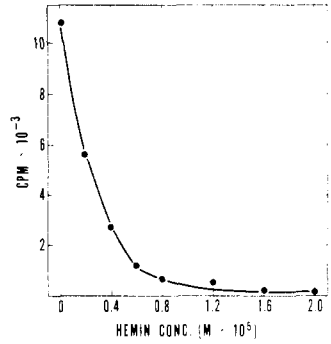

FIGURE 2: Dissociation of template–DNA polymerase complex by hemin. The formation of a [^{14}C]poly[d(A-T)]–DNA polymerase complex was assayed as described under Materials and Methods except for the addition of hemin as indicated.

[^{14}C]poly[d(A-T)] did not protect the DNA–enzyme complex from dissociation by hemin, nor did the synthesis of DNA lead to the stabilization of the DNA–enzyme complex against the action of hemin (data not shown). These results suggest that hemin not only prevents the formation but also causes the dissociation of the DNA–enzyme complex.

Interaction of Hemin with DNA Polymerase. To determine whether the inhibition of DNA synthesis by hemin is due to its interaction with the enzyme or with the DNA template, we investigated whether hemin forms a complex with DNA polymerase or DNA. A mixture of hemin and DNA polymerase was chromatographed on an agarose column and the elution profile compared with that of DNA polymerase and hemin chromatographed separately (Figure 3). Hemin alone is retarded and eluted in a single peak; however, in the presence of the enzyme, hemin is eluted in two well-separated peaks. The first peak appears in the same position as DNA polymerase and the second peak corresponds to the position of free hemin. This suggests that hemin forms a relatively stable complex with the enzyme. Analogous experiments with hemin and template have shown that hemin is retarded and eluted in a single peak either in the presence or absence of DNA (data not presented).

Effects of Known Inhibitors of DNA Synthesis on the Template–Enzyme Complex. Having demonstrated that hemin prevents association and causes dissociation of the

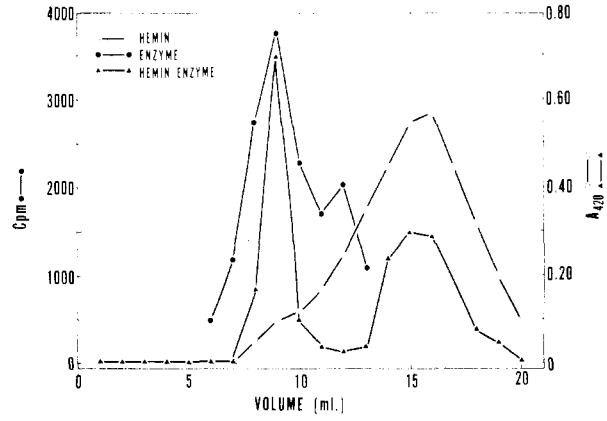

FIGURE 3: Demonstration of the binding of hemin to DNA polymerase by gel filtration on Bio-Gel A-1.5m. Chromatographic procedures and assays for DNA polymerase and hemin are described under Materials and Methods. DNA polymerase alone (●), hemin alone (○), and DNA polymerase and hemin (▲).

Table I: Effects of Inhibitors on Binding of DNA-Dependent DNA Polymerase to Poly[d(A-T)].[a]

Inhibitor	[^{14}C]Poly-[d(A-T)] Bound (cpm)	% Inhib.
Control	10,670	
Hemin (8.0 μM)	1,200	89
Rifamycin AF/013 (40 μg/ml)	600	94
Ethidium bromide (24 μg/ml)	8,310	22
Daunomycin (16 μg/ml)	8,430	21

[a] The reaction mixtures were as described under Materials and Methods except for the addition of various inhibitors as indicated.

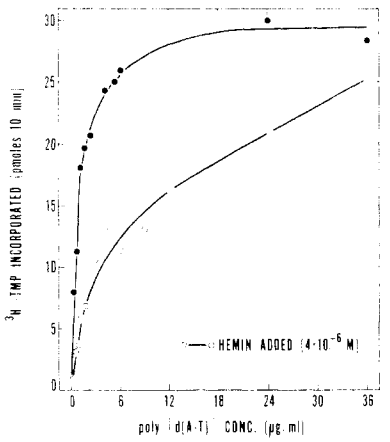

FIGURE 4: The effect of template concentration on the rate of DNA synthesis in the presence (O) and absence (●) of 4×10^{-6} M hemin. Assay conditions were as described under Materials and Methods except that the concentrations of DNA varied from 0.25 to 36 μg/ml.

DNA-enzyme complex by interacting with DNA polymerase, we also investigated the effects of other inhibitors of DNA synthesis on the formation of the template-enzyme complex (Table I). Neither ethidium bromide, an interacalating dye of DNA, nor daunomycin, an anthracycline antibiotic which has a high affinity for DNA and is believed to intercalate between base pairs (Goldberg and Friedman, 1971), has any significant effect on the formation of the template-enzyme complex at concentrations which markedly inhibit DNA synthesis (Byrnes *et al.*, 1973). However, incubation of DNA template-DNA polymerase complex with Rifamycin AF/013, an analog of Rifamycin B, resulted in almost complete dissociation of the template-enzyme complex as measured by retention of the [^{14}C]-poly[d(A-T)]-enzyme complex on a Millipore filter. This suggests that the mechanism of inhibition of DNA synthesis by compounds that interact with the DNA template is not due to the inhibition of binding of DNA polymerase to the DNA template, but is a result of blocking of the movement of the enzyme along the DNA template. Thus, the binding assay could provide a simple and sensitive assay to determine whether inhibitors of DNA synthesis interact with DNA polymerase or DNA template.

Kinetics of Hemin Inhibition of DNA Synthesis. The effect of hemin on the initial rate of DNA synthesis as a function of DNA template concentration is shown in Figure 4. It can be seen that the per cent inhibition of DNA polymerase activity by a fixed concentration of hemin can be reduced by increasing the concentration of DNA. A double-reciprocal plot of the rate of DNA synthesis as a function of DNA concentration in the presence and absence of hemin (Figure 5) shows that the apparent K_m for DNA is increased in the presence of hemin while the V_{max} remains unchanged, suggesting that hemin is a competitive inhibitor with respect to template. Analogous experiments with poly[d(A-T)] as template at a concentration of 0.24 A_{260} unit/ml show that the apparent K_m for dATP and for TTP remains unchanged at 4×10^{-7} and 2×10^{-6} M, respectively, either in the presence or absence of 4×10^{-6} M hemin; however, the V_{max} is reduced from 41 to 19 pmol/10 min in the presence of hemin (data not shown), suggesting that hemin is a noncompetitive inhibitor with respect to substrate.

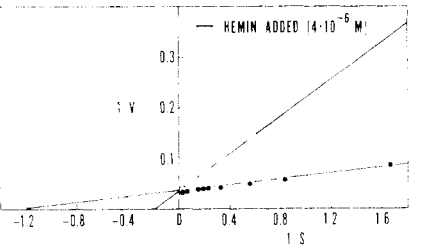

FIGURE 5: Double-reciprocal plot of the data in Figure 4: (O) presence of 4×10^{-6} M hemin; (●) absence of hemin.

Reversibility of Hemin Inhibition. If hemin indeed functions as a physiological regulator of cytoplasmic nucleic acid polymerase activity, then the binding of hemin to DNA polymerase should not lead to irreversible inactivation of the enzyme. The reversibility of hemin inhibition of DNA synthesis is shown in Table II. Addition of 4×10^{-6} M hemin results in about 75% inhibition of DNA synthesis. This inhibition is reversed by the addition of globin, while

Table II: Reversibility of Hemin Inhibition by Human Globin.[a]

Inhibitor Added	Protein Added	[^{3}H]TMP Incorp. (pmol)	% Inhib.
Control		40	0
Hemin (4×10^{-6} M)		11.3	72
	Globin (2.4×10^{-6} M)	41	0
Hemin (4×10^{-6} M)	Globin (2.4×10^{-6} M)[b]	41	0
Hemin (4×10^{-6} M)	Globin (2.4×10^{-6} M)[c]	27	32
	Bence-Jones protein (2.6×10^{-6} M)	40	0
Hemin (4×10^{-6} M)	Bence-Jones protein (2.6×10^{-6} M)	10	75
	Carbonic anhydrase (2.8×10^{-6} M)	40	0
Hemin (4×10^{-6} M)	Carbonic anhydrase (2.8×10^{-6} M)	11	72

[a] Reaction conditions were as described under Materials and Methods except for the addition of hemin and protein as indicated. [b] Globin added before the addition of hemin. [c] Globin added after the addition of hemin.

addition of non-heme-containing proteins, such as Bence-Jones protein or carbonic anhydrase, has no effect on hemin inhibition. This shows that the inhibition of DNA polymerase by hemin is not due to irreversible denaturation of the enzyme and suggests the possibility that hemin coordinates the activities of the cytoplasmic polymerases with the synthesis of globin.

Discussion

Hemin inhibits DNA synthesis with cytoplasmic DNA polymerase by binding to the enzyme and preventing the binding of template. Kinetic analysis of DNA polymerase inhibition by hemin shows that hemin is a competitive inhibitor with respect to template and a noncompetitive inhibitor with respect to substrate, the deoxyribonucleoside triphosphates. This is analogous to the mechanism of hemin inhibition of RNA-dependent RNA polymerase (K. M. Downey *et al.*, unpublished observation). The reversibility of hemin inhibition by equimolar concentrations of globin suggests that the inhibition of DNA polymerase by hemin results from reversible modification of the enzyme affecting the template binding site and is not a consequence of irreversible inactivation of the enzyme. This is consistent with the suggestion that hemin is a physiological regulator of cytoplasmic DNA and RNA polymerase activities.

Since greater than 80% of the protein synthesized in erythroid cells is hemoglobin, it has been suggested that amplification of hemoglobin mRNA occurs in these cells. We have postulated that the cytoplasmic DNA and RNA polymerases are involved in amplification of hemoglobin mRNA (Byrnes *et al.*, 1973; Downey *et al.*, 1973). Hemin, which plays an important role in the regulation of synthesis of both components of hemoglobin in erythroid cells, also controls the activities of erythroid cytoplasmic DNA polymerase and RNA-dependent RNA polymerase. Hemin regulates its own synthesis by feedback inhibition of ALA synthetase (Scholnick *et al.*, 1969; Bottomley and Smithee, 1969) and it is required for continued initiation of globin synthesis (Grayzel *et al.*, 1966; Waxman and Rabinowitz, 1966). The concentration that is required for optimal globin synthesis (1×10^{-5} M) (Bruns and London, 1965) is also the concentration that results in marked inhibition of the activities of ALA synthetase (Scholnick *et al.*, 1969; Kaplan, 1971) and cytoplasmic DNA and RNA polymerases (Byrnes *et al.*, 1973; Downey *et al.*, 1973). This suggests that hemin modulates and coordinates the activities of the various cytoplasmic enzymes involved in globin synthesis.

Hemin may be a more general regulator of cytoplasmic DNA and RNA polymerases and not restricted to erythroid cells, since hemin has been found to stimulate protein synthesis in both erythroid and nonerythroid cells and the synthesis of nonheme proteins (Beuzard *et al.*, 1973). However, whether hemin is a general or specific regulator of DNA and RNA polymerases will have to await further investigation.

Acknowledgment

We thank Mrs. Bonnie Kessler and Mrs. Melca Rodriguez for excellent technical assistance.

References

Aposhian, H. V., and Kornberg, A. (1962), *J. Biol. Chem. 237,* 519.

Baril, E. F., Brown, O. E., Jenkins, M. D., and Laszlo, J. (1971), *Biochemistry 10,* 1971.

Beuzard, Y., Rodvien, R., and London, I. M. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 1022.

Bottomley, S. S., and Smithee, G. A. (1969), *Blood 34,* 857.

Bruns, G. P., and London, I. M. (1965), *Biochem. Biophys. Res. Commun. 18,* 236.

Byrnes, J. J., Downey, K. M., and So, A. G. (1973), *Biochemistry 12,* 4378.

Byrnes, J. J., Downey, K. M., and So, A. G. (1974a), *Nature (London) 248,* 687.

Byrnes, J. J., Downey, K. M., and So, A. G. (1974b), *Proc. Nat. Acad. Sci. U.S. 71,* 205.

Chang, L. M. S., and Bollum, F. J. (1972), *Biochemistry 11,* 1264.

Downey, K. M., Byrnes, J. J., and So, A. G. (1973), *Proc. Nat. Acad. Sci. U.S. 70,* 3600.

Goldberg, I. H., and Friedman, P. A. (1971), *Annu. Rev. Biochem. 40,* 775.

Grayzel, A. I., Hochner, P., and London, I. M. (1966), *Proc. Nat. Acad. Sci. U.S. 55,* 650.

Jones, O. W., and Berg, P. (1966), *J. Mol. Biol. 22,* 199.

Scholnick, P. L., Hammaker, L. E., and Marver, H. S. (1969), *Proc. Nat. Acad. Sci. U.S. 63,* 65.

Sedwick, W. D., Wang, T. S., and Korn, D. (1972), *J. Biol. Chem. 247,* 5026.

Spadari, S., Muller, R., and Weissbach, A. (1974), *J. Biol. Chem. 249,* 299.

Waxman, H. S., and Rabinowitz, M. (1966), *Biochim. Biophys. Acta 129,* 369.

Weissbach, A., Schlabach, A., Fridlender, R., and Bolden, A. (1971), *Nature (London) New Biol. 231,* 167.