Ribonucleotide Reductase from *Thermus* X-1, a Thermophilic Organism†

Gloria N. Sando and Harry P. C. Hogenkamp*

ABSTRACT: A ribonucleotide reductase requiring 5′-deoxy-5′-adenosylcobalamin has been partially purified from cell-free extracts of the extreme thermophile, *Thermus* X-1. These preparations catalyzed the reduction of ribonucleoside triphosphates and required a dithiol and adenosylcobalamin for activity. The optimum temperature of the reaction was approximately 70°. Of the ribonucleoside triphosphates tested, GTP and CTP were the best substrates while UTP and ATP were reduced at only one-tenth the rate of GTP reduction. However, the rate of ATP reduction was specifically enhanced by dGTP and this stimulation was temperature dependent. Maximum stimulation of ATP reduction was observed at approximately 75° while no stimulation could be detected at 37°. Estimates of kinetic constants made at 37 and 70° indicated that the apparent K_m values for the nucleotide substrates increased as the assay temperature was increased.

Ribonucleotide reductase from *Lactobacillus leichmannii* catalyzes the reduction of ribonucleoside triphosphates to the corresponding deoxyribonucleoside triphosphates and has an absolute requirement for adenosylcobalamin[1] as a coenzyme (Blakley, 1966a). This enzyme reduces the nucleotide substrates at markedly different rates; however these rates are selectively modified by deoxyribonucleoside triphosphates which function as specific allosteric activators (Vitols *et al.*, 1967). Earlier results from this laboratory (Follmann and Hogenkamp, 1971) suggest that ribonucleotide reductase from *L. leichmannii* has at least two different effector sites: binding of an effector at one site causes stimulation of substrate reduction, while binding at the other site affects enzyme–coenzyme interaction. More recently Panagou *et al.* (1972) purified ribonucleotide reductase from *L. leichmannii* to homogeneity and demonstrated that the enzyme consists of a single polypeptide chain with a molecular weight of 76,000 daltons. Thus the reductase from *L. leichmannii* appears to be a monomeric enzyme with binding sites for: adenosylcobalamin, ribonucleotide substrate, dithiol substrate, and two nucleotide effectors.

Adenosylcobalamin-dependent ribonucleotide reductases from other microorganisms have not been studied as extensively, but the evidence presented to date suggests that in general the reduction of ribonucleotides is subject to allosteric control by deoxyribonucleotides (Barker, 1972).

A recent survey of the distribution of adenosylcobalamin-dependent ribonucleotide reductases indicated that a cell-free extract of the extreme thermophile, *Thermus aquaticus* YT-1, was able to catalyze tritium exchange between adenosylcobalamin-5′-t_2 and water (Gleason and Hogenkamp, 1972). This exchange reaction serves as a qualitative assay for the adenosylcobalamin-dependent reductases. Thus the ability of the thermophile to grow at elevated temperatures suggests that its ribonucleotide reductase is catalytically active at these temperatures. If the reductase from the thermophile also has effector sites in addition to the sites for nucleotide substrate, dithiol substrate, and coenzyme, this enzyme must not only be able to maintain the integrity of the active site at elevated temperatures but must also possess the flexibility in structure to undergo the conformational changes characteristic of allosteric enzymes. Thus it is of interest to establish the catalytic and regulatory properties of ribonucleotide reductase from an extremely thermophilic organism. This paper describes several properties of a partially purified ribonucleotide reductase from *Thermus* X-1, a thermophilic organism which is closely related to *Thermus aquaticus* but which lacks the carotenoid pigment (Ramaley and Hixson, 1970).

Materials and Methods

Materials. Nucleotides were purchased from P-L Biochemicals. Bovine serum albumin, horse heart cytochrome *c*, type VI, rabbit muscle aldolase, pig heart diaphorase, D,L-lipoic acid, dithiothreitol, dithioerythritol, glutathione, protamine sulfate, phenylmethanesulfonyl fluoride, and 3,3-dimethylglutaric acid were purchased from Sigma. Enzyme grade ammonium sulfate was obtained from Schwarz-Mann. Ribonucleotide reductase from *Lactobacillus leichmannii* was kindly supplied by Dr. R. L. Blakley. Hen ovalbumin was a gift of Dr. R. Montgomery. Thioredoxin and thioredoxin reductase were isolated from *Escherichia coli* by the procedure of Laurent *et al.* (1964) and Moore *et al.* (1964) omitting the final gel filtration step. Dihydrolipoate was prepared by reduction of lipoic acid (Gunsalus and Razzell, 1957) without distillation of the product. Adenosylcobalamin-5′-t_2 (22 Ci/mol) and adenosylcobalamin were prepared as described before (Gleason and Hogenkamp, 1971; Hogenkamp and Pailes, 1968). *Thermus* X-1 was a gift from Dr. R. Ramaley.

Methods. Thermus X-1 was routinely grown in 2800-ml Fernbach flasks containing the following medium: 50 ml of Castenholz 20× basal salts stock solution (Castenholz, 1967), 1.7 g of Bio-Cert tryptone, 1.7 g of BBL trypticase soy broth, 2.5 g of glucose, and 950 ml of water, adjusted to pH 8.2 with 1 N NaOH. The flasks were inoculated with 100 ml of an actively growing culture. The inoculum was prepared from a 3% agar slant of the same medium without added glucose and cultured twice in the liquid medium without glucose. The cultures were grown at 70° in an environmental incubator shaker (New Brunswick Scientific Co., Model G-25) at 300

† From the Department of Biochemistry, College of Medicine, The University of Iowa, Iowa City, Iowa 52242. *Received April 25, 1973.* This work was supported by U. S. Public Health Grants AM-08627 and GM 00550 from the National Institutes of Health.

[1] Abbreviation used is: adenosylcobalamin, 5′-deoxy-5′-adenosylcobalamin.

rpm. The cells were harvested in the logarithmic growth phase (absorbance at 650 nm, 0.8–1.0) by centrifugation at 13,000*g* for 15 min, washed once in 0.03 M potassium dimethylglutarate buffer (pH 7.2), and stored as a cell paste at −10°.

Preparation of Cell-Free Extract. Frozen cell paste (30–50 g) was suspended in two volumes of 0.03 M dimethylglutarate buffer (pH 7.2), containing 1 mM phenylmethanesulfonyl fluoride, and the cells were disrupted by sonic oscillation for 15 min at 8–10° in a Raytheon 10-kcycle sonic oscillator. The cell debris was removed by centrifugation at 40,000*g* for 40 min. The supernatant solution had a specific activity of 1.5 μmol/hr per mg of protein at 70° with GTP as substrate.

Partial Purification of Enzyme. All operations were carried out at 4°. The supernatant was adjusted to pH 5.5 with 2 N acetic acid, and protamine sulfate (10 mg/ml of buffer) was added to a final concentration of 1 mg of protamine sulfate/30 mg of protein. The solution was stirred for 30 min and the precipitate was removed by centrifugation at 28,000*g* for 60 min. The supernatant was adjusted to pH 9 with 3 N ammonium hydroxide, 14.4 g of ammonium sulfate/100 ml of supernatant was added, and after 30 min the mixture was centrifuged at 40,000*g* for 120 min. The precipitate was resuspended in 20 ml of 0.03 M potassium dimethylglutarate buffer (pH 7.2) and 0.56 g of ammonium sulfate was added. After 30 min the precipitate was removed by centrifugation at 28,000*g* for 30 min. Extracts prepared in this manner contained approximately 1 mg of protein/ml. This procedure provides a 10-fold purification (specific activity approximately 15 μmol/hr per mg of protein at 70° with GTP as substrate) with an approximately 30% recovery of enzyme activity. These preparations stored at −10° retain their enzyme activity for at least 1 month.

Assay Procedures. Two methods were used to measure ribonucleotide reductase activity. In the first and more sensitive assay ribonucleotide reductase was determined by measuring tritium exchange between adenosylcobalamin-5′-t_2 and water (Hogenkamp *et al.*, 1968). Reaction mixtures contained 0.18 M potassium dimethylglutarate (pH 7.2), 50 mM dihydrolipoate, 2–10 mM nucleotide, 5 mM $MgCl_2$, 3 mM EDTA, 20 μM adenosylcobalamin-5′-t_2 and approximately 0.02 mg of a partially purified cell-free extract in a total volume of 0.5 ml. The coenzyme was added to the substrate mixture in dim light and the reaction was initiated by the addition of substrate mixture to the enzyme preparation. The assay tubes were incubated in the dark at 37° for 15 min or at 70° for 8 min. The reaction was terminated by freezing in liquid nitrogen and water was removed from the reaction mixtures by sublimation. A 0.1-ml aliquot of the thawed sublimate was added to 10 ml of scintillation fluid (Bray, 1960) and assayed for radioactivity in a Packard Model 3003 Tri-Carb liquid scintillation spectrometer. Specific activity is defined as the radioactivity released to the solvent (counts per minute) per milligram of protein for the 8- or 15-min incubation period.

In the second method the diphenylamine procedure was used to measure the amount of deoxyribonucleotide formed. The composition of the reaction mixture was similar to that described in the exchange assay except that nonradioactive adenosylcobalamin and a higher concentration of enzyme preparation (approximately 0.1 mg/assay) were used. Purine deoxyribonucleotides were measured as described by Blakley (1966b) while pyrimidine deoxyribonucleotides were estimated by the method of Cowles and Evans (1968).

Protein concentration was determined by the method of Lowry *et al.* (1951). Absorbance measurements were made with a Zeiss PMQII spectrophotometer.

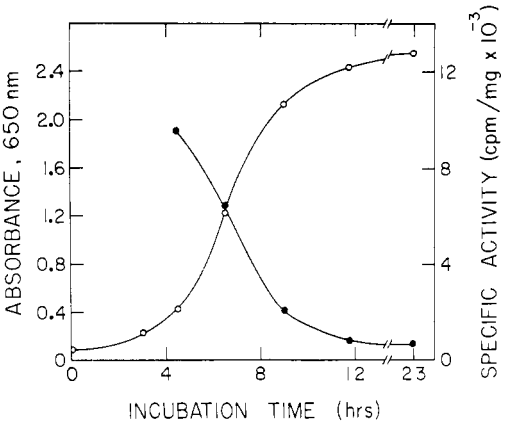

FIGURE 1: Relationship between growth phase and ribonucleotide reductase specific activity. *Thermus* X-1 was grown at 70° and at the designated times 100-ml aliquots were removed. The absorbance at 650 nm (○) was determined and the cells were harvested, washed once, and frozen. Cell-free extracts were prepared as described under Methods. Ribonucleotide reductase activity was measured at 37° with the tritium-exchange method; specific activities are expressed as the radioactivity (counts per minute) released per milligram of protein per 10-min incubation (●).

Results

Effect of Growth Conditions on Ribonucleotide Reductase Activity. In order to determine the period of maximum enzyme production, ribonucleotide reductase activity was measured during the growth of *Thermus* X-1 at 70°. The specific activities of crude extracts from cells harvested at various intervals of the growth curve decreased sharply as the cells approached maximum growth (Figure 1). Extracts from cells harvested in the stationary phase were virtually inactive. The addition of cyanocobalamin, 0.01–1.0 μg/l. of growth medium, had little effect on the growth of the organism or on the specific activity of the reductase.

Effect of Temperature on Catalytic Activity. A comparison of the ribonucleotide reductase activities from *Thermus* X-1 and from *L. leichmannii* over a wide range of temperatures is shown in Figure 2. The optimum temperature for the enzyme derived from the thermophile (70–75°) is approxi-

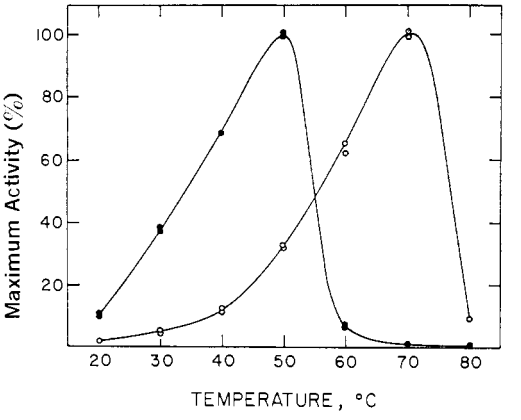

FIGURE 2: Effect of temperature on ribonucleotide reductase activity from *Thermus* X-1 (○) and from *Lactobacillus leichmannii* (●). Reaction mixtures contained 0.3 M potassium phosphate buffer (pH 7.2), 50 mM dihydrolipoate, 20 μM adenosylcobalamin, 2 mM GTP, and 0.1 mg of reductase in a final volume of 0.5 ml. For reaction mixtures containing *Thermus* X-1 reductase 10 mM GTP was used. Reaction mixtures were incubated at the indicated temperatures for 10 min and deoxyribonucleotide formation was measured by the diphenylamine procedure.

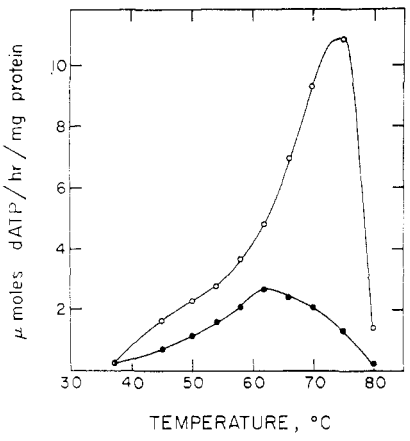

FIGURE 3: Effect of temperature on ATP reduction with and without effector. Reaction mixtures contained either 8 mM ATP (●) or 8 mM ATP and 0.2 mM dGTP (○) and were incubated at the designated temperatures for 8 min. Other assay conditions are described under Methods.

mately 20° higher than the optimum temperature of the reductase from the mesophile. This optimum temperature corresponds very closely to the optimum temperature for growth of *Thermus* X-1 (Ramaley and Hixson, 1970).

Requirements for Ribonucleotide Reductase Activity. The partially purified extracts of *Thermus* X-1 catalyzed the reduction of ribonucleoside triphosphates to the corresponding deoxyribonucleoside triphosphates as well as tritium exchange between adenosylcobalamin-5′-t_2 and water. Both reactions had an absolute requirement for the coenzyme, dihydrolipoate, and a nucleoside triphosphate. Only dithiols, such as dihydrolipoate, dithiothreitol, and dithioerythritol, were able to function as reducing substrates. The thioredoxin–thioredoxin reductase system isolated from *E. coli* was not effective as a reducing substrate at 70°. However, at 37° this reducing system was able to replace dihydrolipoate in the more sensitive tritium-exchange reaction, suggesting that thioredoxin reductase was inactivated at 70°.

Only nucleoside triphosphates were able to serve as substrates or as promoters of the exchange reaction (Table I). The results show that the enzyme from the thermophile is a ribonucleoside triphosphate reductase. Like the reductase from *L. leichmannii*, the enzyme from *Thermus* X-1 reduced the nucleotide substrates at markedly different rates: GTP was the best substrate followed by CTP, ITP, UTP, and ATP. The last nucleotide was reduced at only one-tenth the rate of GTP reduction.

Effect of Deoxyribonucleotides. Ribonucleotide reductase in other microorganisms and in mammalian systems has been shown to be allosterically affected by various deoxyribonucleotides. The results presented in Table II show that ribonucleotide reductase from *Thermus* X-1 was also affected by deoxyribonucleotides. The most striking effect was the fourfold increase in the rate of ATP reduction with the addition of dGTP. Other less striking effects were the inhibition of CTP and UTP reduction by dGTP. The effect of dGTP on ATP reduction was temperature dependent (Figure 3). Little or no increase in the rate of ATP reduction was observed until the temperature of the reaction mixture was raised to approximately 55°. At temperatures beyond 55° the stimulatory effect of dGTP became more pronounced with a maximum stimulation at approximately 75°. The presence of dGTP not only increased the rate of ATP reduction but it also increased the temperature optimum from about 60 to about 75°.

Stability. Partially purified preparations of ribonucleotide reductase in 0.03 M dimethylglutarate buffer (pH 7.2) remained fully active on storage at room temperature for 24 hr. Indeed little activity was lost upon incubation of the partially purified enzyme at 50 or 70° for at least 1 hr. On the other hand incubation of the enzyme preparation at 80° caused rapid inactivation (Figure 4A). In contrast to many other enzymes which appear to be stabilized by the addition of substrate and/or coenzyme, the addition of dihydrolipoate, coenzyme, and nucleotide substrate or nucleotide effector caused more rapid inactivation of the reductase at 70° (Figure 4B). Incubation of the reductase preparation in a complete reaction mixture at 70° resulted in loss of enzyme activity. If it is assumed that the enzyme was inactivated by a first-order process the amount of product formed in a complete reaction mixture as a function of time can be shown to be: $P = V_0(1 - e^{-kt})k^{-1}$, where V_0 is the maximum velocity at zero time and k is the first-order inactivation constant for the

TABLE I: Nucleotide Specificity of *Thermus* X-1 Ribonucleotide Reductase.[a]

Nucleotide	Reduction (μmol/hr per mg of Protein)	Tritium Exchange (cpm/mg of Protein) × 10^{-2}
GTP	15.4	11,988
GDP	2.1	1,898
GMP	0	0
CTP	10.7	9,690
CDP	2.7	842
CMP	1.9	11
ITP	7.6	6,225
IDP	0.5	563
UTP	2.1	2,800
UDP	0.8	400
UMP	0.2	0
ATP	1.2	1,373
ADP	0.06	84
AMP	0	0

[a] Assay conditions are described under Methods. Reaction mixtures contained 10 mM nucleotide and were incubated at 70° for 8 min.

TABLE II: Effect of Deoxyribonucleotides on *Thermus* X-1 Ribonucleotide Reductase.[a]

Nucleotide	μmol of Deoxynucleotide/mg of Protein per hr				
	None	dGTP	dCTP	dUTP	dATP
GTP	11.2	10.4	10.7	13.9	10.2
CTP	7.8	4.6	7.5	8.1	7.4
UTP	1.7	0.6	1.5	1.4	1.4
ATP	1.3	5.2	0.9	2.0	1.0

[a] Assay conditions are described under Methods. Reaction mixtures containing 8 mM ribonucleotide and 0.2 mM deoxyribonucleotide were incubated at 70° for 8 min.

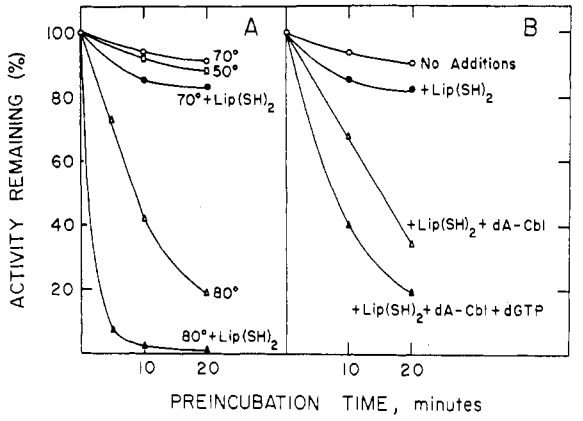

FIGURE 4: Thermostability of *Thermus* X-1 ribonucleotide reductase. Partially purified enzyme preparations were preincubated at the designated temperatures, and at the indicated time intervals aliquots were removed and assayed for enzymatic activity with GTP as substrate. Where indicated the preincubation mixtures contained 0.05 M dihydrolipoate, 20 μM adenosylcobalamin, and 0.2 mM dGTP. All reactions shown in B were run at 70°.

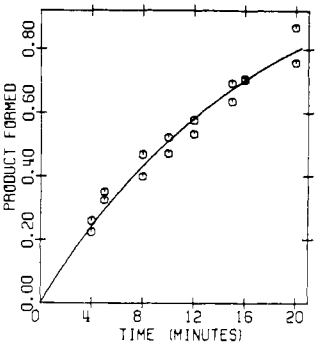

FIGURE 5: Progress curve for *Thermus* X-1 ribonucleotide reductase. Reaction mixtures contained 2 mM GTP and were incubated at 70° for the indicated time intervals. Other assay conditions are described under Methods. The solid curve represents a least-squares fit of the experimental data (open circles) to the equation $P = V_0(1 - e^{-kt})k^{-1}$. The data points were given equal weights. The square of the difference between the observed and calculated product concentrations was minimized by means of a computer program (Los Alamos Publication LA-2367 and Addenda). The present modification was written in Fortran IV by Dr. G. Gordon, and the enzyme kinetic subroutines were written by Dr. K. Sando, both of the University of Iowa.

enzyme. If the experimental data (product formed at different time intervals) are subjected to a nonlinear least-squares fit to this equation (Figure 5) values for k and V_0 are obtained. This value for k agrees very well with the first-order inactivation constant obtained from the stability studies (Figure 4B). It is clear from Figure 5 that the rate of product formation is not constant during the 8-min incubation period. However if the nonlinearity at 70° is due only to the first-order degradation of the enzyme, the enzyme assay is valid because the enzyme activity calculated from the product formed after 8 min is a constant fraction of the true activity. At 37° the rate of product formation was constant for at least 30 min.

Effects of Divalent Cations. Ribonucleotide reductase from *Thermus* X-1 did not show an absolute requirement for a divalent metal ion; however both Mg^{2+} and Mn^{2+} stimulated enzyme activity. The results presented in Figure 6 suggest that the divalent ion–nucleotide complexes are the preferred substrates. Maximum stimulation was observed at the metal concentration equimolar with the nucleotide; at higher concentrations both Mg^{2+} and Mn^{2+} inhibited ribonucleotide reduction. Concentrations of EDTA up to 20 mM had little effect on enzyme activity.

Effects of pH. The enzyme showed maximum activity between pH 7.8 and 8.4 at 37° as well as at 70°. At 70° enzyme activity dropped sharply at pH values greater than 8.4.

Estimation of Molecular Weight. An approximate value for the molecular weight of the enzyme was obtained by gel filtration of the partially purified extract on Sephadex G-200. Rabbit muscle aldolase (Taylor *et al.*, 1956), pig heart diaphorase (Massey *et al.*, 1962), ovalbumin (Montgomery, 1970), cytochrome *c* (Margoliash, 1962), and *L. leichmannii* ribonucleotide reductase (Panagou *et al.*, 1972) were used as calibration standards. A comparison of the elution volume of the reductase from the thermophile with the elution volumes of the standard proteins indicated a molecular weight of approximately 80,000.

Kinetic Constants. As shown in Table I ribonucleotide reductase from *Thermus* X-1 reduced the ribonucleotide substrates at different rates. Double-reciprocal plots of reaction velocity at 37 or 70° against substrate concentration for the best substrates, GTP and CTP, showed marked substrate inhibition over the concentration range studied (0.2–10 mM) and thus apparent K_m values could not be accurately determined (Figure 7). However, estimates were made by a nonlinear least-squares fit of the velocities at concentrations less than 2 mM to the Michaelis–Menten equation. In the presence of saturating coenzyme and dihydrolipoate concentrations (20 μM and 50 mM, respectively) apparent K_m values for GTP and CTP were 0.99 ± 0.19 and 1.4 ± 0.3 mM, respectively, at 70°, while at 37° these constants were found to be lower (0.29 ± 0.03 and 0.32 ± 0.02 mM). Double-reciprocal plots of reaction velocity against substrate concentration for the poorer substrates, ATP and UTP, were not linear, and no apparent K_m values could be determined. A double-reciprocal plot for reaction velocity against ATP concentration in the presence of 0.2 mM dGTP (Figure 7) shows a behavior similar to that of GTP and CTP with substrate inhibition at high concentrations of ATP. The apparent K_m for ATP estimated as described above was 1.0 ± 0.2 mM at 70°. A linear double-reciprocal plot of reaction velocity against dGTP concentrations was obtained in the presence of 8 mM ATP and 20 μM adenosylcobalamin. From the data an apparent K_m of 48 ± 9 μM for dGTP as an effector was calculated. The apparent K_m for adenosylcobalamin depended not only on the concentra-

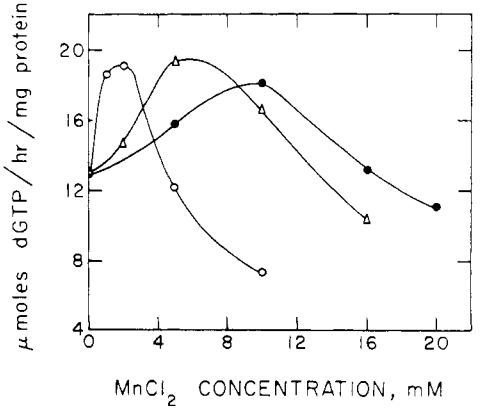

FIGURE 6: Effect of divalent cation concentration on ribonucleotide reductase activity. Assay conditions are described under Methods. Reaction mixtures contained 2 mM (○), 6 mM (△), and 10 mM (●) GTP, EDTA was omitted, and dithiothreitol (50 mM) was used as the dithiol.

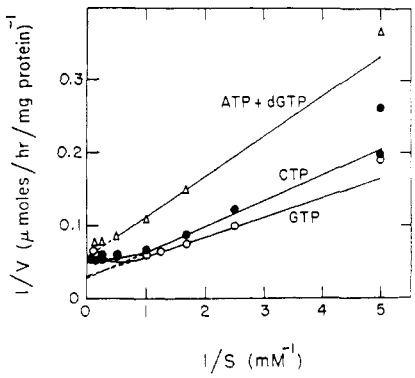

FIGURE 7: Dependence of the rate of reduction on nucleotide concentration. Reaction mixtures contained 0.18 M potasium dimethylglutarate (pH 7.2), 50 mM dihydrolipoate, 3 mM EDTA, 20 μM adenosylcobalamin, and 0.1 mg of the partially purified extract in a total volume of 0.5 ml. GTP, CTP, and ATP were added at the indicated concentrations; dGTP concentration was 0.2 mM. Reaction mixtures were incubated at 70° for 8 min and deoxyribonucleotide formation was measured with the diphenylamine reaction. The values for the kinetic parameters were calculated by means of a computer program in which the square of the difference between the observed and calculated initial velocities is minimized. (See Figure 5 for further details.)

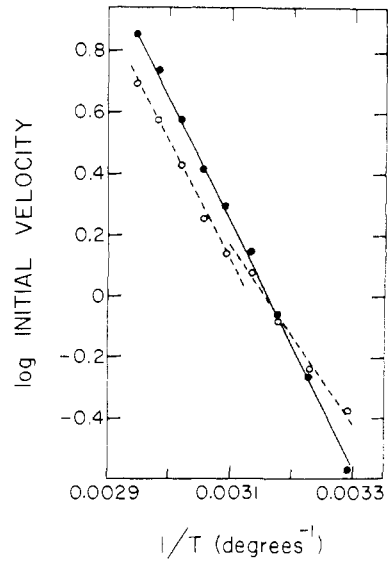

FIGURE 8: Arrhenius plots of *Thermus* X-1 ribonucleotide reductase with GTP (solid circles) and ATP and dGTP (open circles) as substrates. Reaction mixtures contained 10 mM GTP or 8 mM ATP and 0.2 mM dGTP and were incubated at the designated temperatures for 8 min. Other assay conditions are described under Methods.

tion of the nucleotide substrate but also on the nature of the nucleotide. In the presence of saturating concentrations of GTP (10 mM) the apparent K_m of the coenzyme was 3.2 ± 0.1 μM while in the presence of 10 mM ATP and 0.2 mM dGTP the apparent K_m of the coenzyme decreased to 0.65 ± 0.03 μM. No apparent K_m for the coenzyme could be calculated in the presence of ATP without effector because double-reciprocal plots were not linear.

Discussion

Adenosylcobalamin-dependent ribonucleotide reductases have been isolated from *Lactobacillus leichmannii* (Blakley, 1966a), *Euglena gracilis* (Gleason and Hogenkamp, 1970) and *Rhizobium meliloti* (Cowles and Evans, 1968). Whereas the enzymes from the first two organisms use ribonucleoside triphosphates as substrates, ribonuleoside diphosphates are the preferred substrates for the reductase from *R. meliloti*. Ribonucleotide reductase from the extreme thermophile *Thermus* X-1 resembles most closely the reductase from *L. leichmannii*. Both enzymes utilize ribonucleoside triphosphates and dithiols as substrates and show an absolute requirement for adenosylcobalamin as a coenzyme. Both enzymes reduce the ribonucleotide substrates at markedly different rates and in the presence of specific deoxyribonucleotides the reductases are stimulated. Recently it has been shown that ribonucleotide reductase from *L. leichmannii* is a monomeric enzyme with a molecular weight of 76,000 daltons (Panagou *et al.*, 1972). The molecular weight of the enzyme from *Thermus* X-1 was estimated at approximately 80,000 daltons but no information about the subunit structure is yet available. Ribonucleotide reductase from the thermophile *Thermus* X-1 has a temperature optimum near 70°, almost 20° higher than that of the mesophile. Thus a major difference between these two reductases appears to be the thermostability of the enzyme from *Thermus* X-1. This is most likely an intrinsic property of the enzyme and not due to stabilizing factors in the preparation; however, the latter possibility cannot be eliminated until the enzyme is purified to homogeneity. The presence of nonspecific stabilizing factors is unlikely since the partially purified preparation from the thermophile does not impart thermostability to the purified reductase from *L. leichmannii*.

Unlike the reductase of *L. leichmannii*, the enzyme of *Thermus* X-1 reduces GTP and CTP at similar rates, while ATP is reduced at only one-tenth of the rate of GTP reduction. The allosteric control of ribonucleotide reduction in the thermophile appears to involve predominantly the stimulation of ATP reduction by dGTP. The concentration of the product of the best substrate controls the reduction of the poorest substrate. As is shown in Figure 3 this stimulation by dGTP only becomes effective at temperatures higher than about 55°. Below this temperature dGTP does not function as an allosteric activator. Maximum stimulation of ATP reduction is reached at approximately 70–75°, the optimum temperature for growth of the organism, and below this optimum temperature the allosteric regulation by dGTP becomes much less effective. Similar observations have been made by Orengo and Saunders (1972) who reported that feedback inhibition of pyrimidine ribonucleoside kinase from the thermophile *B. stearothermophilus* by CTP became less effective as the temperature was lowered from that required for maximum growth. In contrast L-threonine deaminase from *B. stearothermophilus* (Thomas and Kuramitsu, 1971) became less sensitive to feedback inhibition by L-isoleucine as the assay temperature was increased from 30 to 70°.

Although the apparent K_m values for GTP and CTP are only approximate because both nucleotides inhibit at high concentrations, the results suggest that the apparent K_m values of the nucleotides increase as the assay temperature is increased and thus at higher temperatures higher concentrations of nucleotide are required to saturate the reductase. Similar effects of temperature on the K_m values have been shown for several enzymes (Hochachka and Somero, 1971; Himes and Wilder, 1968; Helmreich and Cori, 1964; Iwatsuki and Okazaki, 1967).

The effect of temperature on the catalytic process of the enzyme is presented in Figure 8 in the form of Arrhenius plots. With GTP as the nucleotide substrate a linear relation-

ship between the logarithm of velocity and the reciprocal absolute temperature was obtained. The activation energy for the reduction of GTP calculated from these data is 18.6 cal/mol. In contrast, with ATP as the nucleotide substrate and dGTP as its allosteric effector the Arrhenius plot was not linear. This plot has a marked break between 45 and 50° and the data are best fitted to two separate straight lines which are displaced along the abscissa in this temperature range. The energy of activation calculated from the slope of the line below 45° is approximately 13.3 cal/mol, while at temperatures above 50° the slope of the line (activation energy 17.9 cal/mol) is virtually identical with the slope of the Arrhenius plot with GTP as substrate. Thus the energy of activation is higher at temperatures above 50°. The change to a higher energy of activation at higher temperatures is unusual (Dixon and Webb, 1964); Massey (1953) reported a similar upward bend in the Arrhenius plot for fumarase at alkaline pH and suggested that at higher temperatures fumarase dissociated into subunits with a higher energy of activation. Usually a downward bend to the lower energies of activation is observed; for instance, Orengo and Saunders (1972) reported such an Arrhenius plot for pyrimidine ribonucleoside kinase from *B. stearothermophilus* with a break at 46°. Massey *et al.* (1966) have suggested that a sharp transition between two linear regions of an Arrhenius plot is indicative of a conformational change in an enzyme, both conformers being catalytically active but differing in energies of activation. The linear Arrhenius plot with GTP as substrate suggests that only one form of the enzyme is present between 30 and 70°. The specific stimulation of ATP reduction by dGTP occurs only at temperatures above approximately 55°. Both ATP and dGTP are able to bind to the enzyme at lower temperatures because ATP is reduced, albeit at a very slow rate, and dGTP alone is able to promote tritium exchange between adenosylcobalamin-5′-t_2 and water at 37°. These observations suggest that in the temperature range from 45 to 50° and in the presence of dGTP the enzyme may undergo a specific conformational change which increases the affinity of the enzyme for ATP and/or accelerates a rate-limiting step in the catalytic process. This conformational change is not only temperature dependent but also nucleotide dependent, because only dGTP serves as the activator and only the reduction of ATP is stimulated.

The theoretical Arrhenius plots shown in Figure 9 were obtained by assuming that the enzyme is able to exist in two active forms; at low temperatures the conformer with an activation energy for catalysis of 15 cal/mol predominates, while at high temperatures the other conformer with an activation energy of 18.5 cal/mol predominates. It was also assumed that the two conformers are at equilibrium and that at the transition temperature an equal amount of each conformer is present. In each Arrhenius plot a different value for the ΔH of the transition between the two conformers was assumed. These values for ΔH and for the activation energies were used in the following equation

$$\frac{V_{max}}{E_T} = \frac{Ae^{-E_a/RT} + A'e^{-E'_a/RT}\, e^{-(\Delta H - T\Delta S)/RT}}{1 + e^{-(\Delta H - T\Delta S)/RT}}$$

where V_{max} is the maximum velocity, E_T is the total enzyme concentration, A and A' are the Arrhenius constants, E_a and E'_a are the activation energies for catalysis, ΔS is the entropy change for the transition, R is the gas constant, and T is the absolute temperature. These theoretical plots agree reasonably well with the experimental data and suggest that a conformational change is a possible explanation for the temperature-dependent stimulation of ATP reduction by dGTP. Since the agreement between the theoretical and experimental points improved as the ΔH value was increased from 50 to 200 cal per mol, the ΔH of this thermal transition is probably quite large.

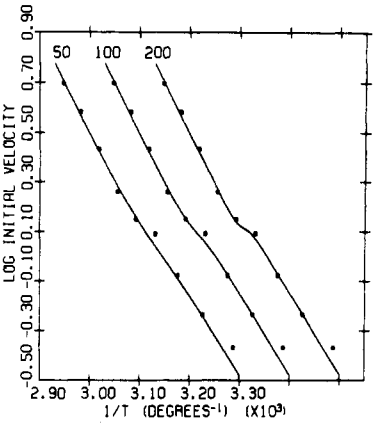

FIGURE 9: Theoretical Arrhenius plots assuming that the enzyme exists in two catalytically active forms with activation energies of 15.0 and 18.5 cal/mol, respectively. The midpoint temperature of the transition between the two conformers was taken to be 49°. The ΔH of the transition between the two conformers was assumed to be 50, 100, and 200 cal per mol. The experimental points (closed circles) taken from Figure 8 are superimposed on each plot.

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Magnetic Resonance Studies of Substrate and Inhibitor Binding to Porcine Muscle Adenylate Kinase†

Nicholas C. Price,‡ George H. Reed,§ and Mildred Cohn*,¶

ABSTRACT: Nuclear magnetic relaxation and electron paramagnetic reasonance (epr) techniques have been used to examine binding of substrates and inhibitors to porcine muscle adenylate kinase. The results show that there is one binding site for MnATP or ATP per mole of enzyme, with dissociation constants of 45 and 35 μM, respectively. The binding parameters for dATP are essentially identical with those of ATP, and the water proton relaxation enhancement for the ternary complexes with ATP and dATP are also similar ($\sim$15 at 24.3 MHz). The dissociation constants for Mn(II) complexes of GTP and tripolyphosphate are an order of magnitude higher than that of MnATP. Ternary complexes of GTP and tripolyphosphate also gave much lower water proton relaxation enhancements than did ATP and dATP. Diadenosine pentaphosphate (Ap_5A) forms a tight complex with the enzyme with a dissociation constant of 1.5 μM; the dissociation constant of the $MnAp_5A$ complex from the enzyme is even smaller (<0.5 μM). Ap_5A is a potent inhibitor of adenylate kinase: 50% inhibition occurs at an Ap_5A concentration of 0.2 μM in the presence of $MnCl_2$. The epr spectrum of the ternary complex, enzyme–MnATP, resembles that of MnATP. Addition of AMP to give the equilibrium mixture results in a considerable broadening of the epr spectrum. On the other hand, the epr spectrum of $MnAp_5A$ changes markedly upon addition of the enzyme, giving rise to a spectrum which resembles that of the equilibrium mixture.

Adenylate kinase is a widely occurring enzyme which catalyzes the following reaction

$$2ADP \overset{M^{2+}}{\rightleftharpoons} ATP + AMP$$

The enzyme is important in maintaining equilibrium among the various species in the adenine nucleotide pool (Noda, 1962). The adenylate kinase reaction is presumed to be a major pathway for phosphorylation of AMP to the level of ADP in the cell.

The interaction of nucleotide substrates with adenylate kinase from rabbit muscle has been examined previously by ultracentrifugation (Kuby *et al.*, 1968) and magnetic resonance techniques (O'Sullivan and Noda, 1968). However, because of the multiplicity of equilibria involved in this system, uncertainties remain in both the dissociation constants and binding stoichiometries. In particular, earlier magnetic resonance studies (O'Sullivan and Noda, 1968) covered only a limited portion of the total ligand saturation curve. A subsequent numerical analysis of these data (Reed *et al.*, 1970) showed that one could not establish the number of binding sites for MnATP from the limited data. We have therefore carried out PRR[1] measurements with the porcine muscle enzyme over a much wider range of nucleotide concentration to enable an unequivocal analysis of the dissociation constants and binding stoichiometries for substrates. We have also investigated the enzyme's interaction with the potent in-

† From the Department of Biophysics and Physical Biochemistry, the University of Pennsylvania, School of Medicine, Philadelphia, Pennsylvania 19174. *Received March 16, 1973.* This work was supported in part by grants from the U. S. Public Health Service, National Institutes of Health GM 12446, and the National Science Foundation GB 32168.

‡ Harkness Fellow of the Commonwealth Fund of New York, 1971–1972. Present address: Department of Biochemistry, Oxford University, Oxford OX1 3QU, England.

§ Recipient of Career Development Award K4-AM 70134 from the National Institute of Arthritis, Metabolic and Digestive Diseases.

¶ Career Investigator of the American Heart Association.

[1] Abbreviations used are: PRR, proton relaxation rate; Ap_5A, P^1,P^5-di(adenosine-5′) pentaphosphate; PSTD, per cent relative standard deviation; PPP, tripolyphosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Ap_4A, P^1,P^4-di(adenosine-5′) tetraphosphate.