Regulatory Role of Adenosine Triphosphate on Hog Kidney *N*-Acetyl-D-glucosamine 2-Epimerase*

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ABSTRACT: Hog kidney *N*-acetyl-D-glucosamine 2-epimerase activity of an enzyme was demonstrated in the absence of adenosine triphosphate (ATP) by using high enzyme and substrate concentration. The ATP-dependent and ATP-independent epimerase activities could not be separated, and the ratio of the specific activities remained nearly constant (≃20) throughout the range of 1500-fold purification of the enzyme. A stimulatory role of ATP on 2-epimerase was demonstrated. The Michaelis constant, K_m, for *N*-acetylmannosamine was 9 mM in the absence of ATP, and 1.7 mM in the presence of saturating concentration of ATP. pH optima for ATP-dependent activity and ATP-independent activity were both 7.4. The ATP saturation curve of the 2-epimerase which was sigmoidal in shape suggested cooperative binding of ATP molecules. Hill plot yielded an interaction coefficient of n = 2.6. Other kinetic evidence suggested the presence of homotropic interactions between substrate molecules. The allosteric effect of ATP was confirmed since specific disruption of the ATP binding site was possible without affecting the ATP-independent catalytic activity of the enzyme. The desensitization of 2-epimerase to ATP requirement was brought about by controlled heat and by *p*-mercuribenzoate treatment. The selective destruction of ATP effect on 2-epimerase strongly suggested that the site for binding the stimulator, ATP is largely independent of the site for binding the substrates.

Ghosh and Roseman (1965) reported an ATP-dependent epimerase in animal tissues which catalyzes the interconversion of *N*-AcGm[1] and *N*-AcMm

$$\text{N-AcGm} \underset{\text{2-epimerase}}{\overset{\text{ATP}}{\longleftrightarrow}} \text{N-AcMm}$$

These investigators purified the enzyme 2-epimerase about 318-fold from hog kidney extracts and studied some of its kinetic properties. Further, they reported that ATP was neither a direct participant in the reaction nor it was degraded by the enzyme during the course of the reaction. The role of ATP was, at that time, obscure. Attempts were made in this laboratory to purify this enzyme further and to study its properties with special reference to the role of ATP. Kinetic studies to be described in this communication suggest that though ATP is not absolutely essential for the activity of 2-epimerase, it exerts a stimulatory effect on the enzyme causing approximately a 20-fold increase of its activity. Evidence is also presented to suggest that the 2-epimerase, like a regulatory enzyme, possesses two distinct but mutually interacting sites—a catalytic site that binds the substrate and an "allosteric" site that binds the effector molecule, ATP. ATP appears to increase the V_{max} and enhance simultaneously the affinity of the enzyme for its substrate, and the cooperativity of substrate binding with the enzyme molecule.

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[1] Abbreviations used are: *N*-AcGm, 2-acetylamino-2-deoxy-D-glucose; N-AcMm, 2-acetylamino-2-deoxy-D-mannose; PEP, phosphoenolpyruvate; PMB, *p*-mercuribenzoate; CMP-sialic acid, cytidine-5′-phosphosialic acid.

Materials and Methods

Preparation of N-Acetylglucosamine Kinase. *N*-Acetylglucosamine kinase, used in the assay of 2-epimerase, was prepared from hog spleen by using the following operations. Hog spleen (100 g) was homogenized with 200 ml of 0.03 M potassium phosphate buffer (pH 7.6) containing 0.001 M EDTA and 0.01 M 2-mercaptoethanol in a Waring blender. The supernatant (150 ml), collected by centrifugation at 16,000*g* for 30 min, was diluted with an equal volume of water and the enzyme was precipitated by adding 22.5 ml of a 2% protamine sulfate solution. The residue collected by centrifugation was washed with 75 ml of 0.05 M potassium phosphate buffer (pH 7.6). The enzyme was extracted from the residue with 75 ml each of 0.075, 0.075, and 0.1 M of the same buffer (pH 7.6). These three extracts were then combined, brought to 32.5% saturation with solid ammonium sulfate, and any precipitate formed at this stage was rejected by centrifugation. The supernatant was brought to a saturation level of 50% with solid ammonium sulfate and the precipitate formed was collected by centrifugation, and dissolved in 10 ml of 0.02 M potassium phosphate buffer (pH 7.6). This concentrated *N*-acetylglucosamine kinase fraction contained some ATPase activity, which was removed by heating this fraction at 60° for 2 min. Coagulated proteins were removed by centrifugation and the supernatant fluid, dispensed in several small test tubes, was kept frozen until used. These enzyme fractions were free of ATPase (Pullman *et al.*, 1960) and other interfering enzymes such as *N*-acetylmannosamine 2-epimerase (Ghosh and Roseman, 1965) and *N*-acetylmannosamine kinase (Kundig and Roseman, 1966) and were quite active for the assay of the 2-epimerase.

Other Materials. ATP, PEP, NADH, AcGm, protamine sulfate, and DEAE-cellulose were obtained from Sigma Chemical Co., St. Louis, Mo. *N*-AcMm was prepared by the

method of Carroll and Conforth (1960) and was found to be electrophoretically pure by the procedure of Jourdian and Roseman (1962). Calcium phosphate gel was prepared by the procedure of Keilin and Hartree (1938). Pyruvate kinase was isolated from goat muscle (Bucher and Pfleiderer, 1955) and was found to be free from ATPase and other interferring enzymes such as *N*-acetylmannosamine kinase, *N*-acetylglucosamine kinase, and *N*-acetylglucosamine 2-epimerase.

Epimerase Assay. The epimerase acitvity was assayed by coupling with specific kinase. The assay consisted of: (1) incubation of the 2-epimerase with *N*-AcMm (and ATP, if necessary), (2) treatment of the reaction products with an excess of the specific *N*-AcGm kinase (ATPase free) and ATP for the complete conversion of any *N*-AcGm formed in step 1 into *N*-AcGm-6-p and the formation of an equivalent amount of ADP, and (3) the estimation of ADP enzymatically by the pyruvate kinase–lactic dehydrogenase assay procedure (Kornberg and Pricer, 1951). Since ATP is not degraded[2] in step 1, the amount estimated in the step 3 equals the amount of *N*-AcGm formed by 2 epimerization.

The incubation mixture for measuring the ATP-independent enzyme activity contained the following components (in micromoles) in a final volume of 0.25 ml: *N*-AcMm, 10.0; Tris-HCl (pH 7.4), 12.5; $MgCl_2$, 2.5; and enzyme fraction. In the measurement of the ATP-dependent activity the incubation mixture contained the following (in micromoles) in a final volume of 0.25 ml: *N*-AcMm, 2.0; ATP, 1.0; Tris-HCl, (pH 7.4), 12.5; $MgCl_2$, 2.5; and enzyme fraction. After incubation at 37° for 30 min, the reaction was stopped by heating at 100° for 1 min. Each assay mixture was treated with approximately 2 units (1 unit of enzyme activity catalyzes the phosphorylation of 1 μmole of *N*-AcGm under the assay conditions described above) of specific *N*-AcGm kinase fraction (freed of ATPase and other interfering enzymes) and 1.0 μmole of ATP (when ATP was not added in step 1). The volume of the mixture was made up to 0.35 ml and incubated again at 37° until all *N*-AcGm was converted into its 6-phosphate ester (usually in less than 15 min). The mixture was heated again 1 min at 100°, cooled, and the volume was made up to 1.0 ml by adding water. Aliquots of this diluted mixture (0.1 ml) were assayed enzymatically for the quantitative estimation of ADP formed in each assay tube based on the lactic dehydrogenase–pyruvate kinase procedure of Kornberg and Pricer (1951). Control tubes used routinely for each set of assay lacked either enzyme fraction or *N*-AcMm in the first incubation mixture. The missing component was, however, added to the control tube immediately before stopping the reaction.

After termination of the reaction (without 2-epimerase) by heating, the conversion of *N*-acetylmannosamine into *N*-acetylglucosamine was routinely monitored (see Methods). In no case was any conversion detected.

The sensitivity of the coupling system was such that as little as 0.01 μmole of *N*-acetylglucosamine could be measured by the production of ADP. The incubation mixture without either 2-epimerase, kinase, or ATP gave insignificant blank values. It was also determined that *N*-acetylglucosamine added to the full incubation mixture without 2-epimerase was fully converted into *N*-acetylglucosamine-6-P and ADP.

Specific activity (both ATP dependent and ATP independent) has been defined as micromoles of *N*-AcGm formed per minute per milligram protein under the assay conditions described above.

One unit of ATP-dependent enzyme activity catalyzes the epimerization of 1 μmole of *N*-AcMm in 1 min under the assay conditions stated above.

Other Assays. Protein was determined by the method of Lowry *et al.* (1951). Amino sugars were estimated by a modified procedure of Reissig *et al.* (1955). ATPase was assayed by the method of Pullman *et al.* (1960); *N*-AcMm kinase by the method of Kundig and Roseman (1966); ATP was assayed by the method of Strehler and McElroy (1957).

Results

Purification of N-AcGm-2-epimerase. Unless otherwise stated, all operations were conducted at temperatures between 0 and 4° and all buffer systems used in the fractionation procedure contained 0.001 M EDTA and 0.01 M 2-mercaptoethanol. The first two steps of the purification procedure, the preparation of crude extract and of protamine extract, were almost similar to those as described previously by Ghosh and Roseman (1964).

CRUDE EXTRACT AND PROTAMINE EXTRACT. Kidney cortex (100 g) was homogenized in a Waring blender with 200 ml of 0.03 M potassium phosphate buffer (pH 7.6) and the supernatant was collected after centrifugation at 16,000*g* for 30 min (crude extract). The crude extract (150 ml) was diluted with equal volume of cold distilled water and treated slowly with 4.5 ml of a 2% protamine sulfate solution with constant stirring; the precipitate formed at this stage was discarded by centrifugation. The supernatant was further treated with 15 ml of a 2% protamine sulfate and the precipitate formed was collected by centrifugation. After preliminary washing with 100 ml of 0.01 M potassium phosphate buffer (pH 7.6), the active enzyme was extracted from the precipitate twice with 45 ml of 0.025 M potassium phosphate buffer (pH 7.6).

BENTONITE ADSORPTION. The combined extracts of protamine precipitate (90 ml) were gently stirred for 10 min with 900 mg of bentonite (suspended in 10 ml of 0.001 M EDTA) and the supernatant was collected by centrifugation. Concentrations of the 2-epimerase in the bentonite supernatant and the protamine extract fractions were very low to permit satisfactory assay of the ATP-independent enzyme activity. The enzyme in the supernatant was next concentrated by adsorbing the enzyme on a small DEAE-cellulose column and eluting it with 0.2 M potassium phosphate buffer (pH 7.6; see DEAE-cellulose step). The concentrated enzyme eluates were dialyzed for 24 hr against 5 mM potassium phosphate buffer (pH 7.6) and designated as "protamine concentrate" and "bentonite supernatant concentrate."

DEAE-CELLULOSE. Bentonite supernatant fraction (100 ml) was applied to a DEAE-cellulose column (1.5 × 10 cm) which had been previously equilibrated with 0.02 M KCl–0.01 M potassium phosphate buffer (pH 7.6). After washing the column with 200 ml of 0.05 M potassium phosphate buffer (pH 7.6), the enzyme was eluted using linear gradient of 0.05–0.15 M potassium phosphate buffer (pH 7.6) containing 20 mM KCl, 1 mM EDTA, and 10 mM 2-mercaptoethanol at flow rate of

[2] It was reported previously (Ghosh and Roseman, 1965) that ATP is not degraded during incubation with 2-epimerase. This observation was confirmed by the assay of ATP (Strehler and McElroy, 1957; Crane and Sols, 1955).

TABLE I: Purification of *N*-Acetyl-D-glucosamine 2-Epimerase.[a]

Fractions	Total Act. Units[c]	Total Act. Yield (%)	Sp Act. ATP Dependent A	Sp Act. ATP Independent B	Ratio of Sp Act. A:B	Purifcn Factor
Crude extract[b]	20	100	0.004			1
Protamine concentrate	16	80	0.103	0.0053	19.4	26
Bentonite concentrate	16	80	1.00	0.05	20	250
DEAE-cellulose eluate	9.3	46.6	5.8	0.29	20	1450
Calcium phosphate gel	7.5	37.6	6.0	0.3	20	1500

[a] The composition of incubation mixtures and the other assay conditions were the same as described in the text. [b] The assay procedure described above was not suitable for measuring the enzyme activity of crude extracts. For the assay of impure preparations the second incubation mixture was treated with $ZnSO_4$ and $Ba(OH)_2$ solutions for the quanitative removal of *N*-AcGm-6-P (Somogyi, 1945). The amount of *N*-AcMm disappearing due to epimerization was then estimated by the Morgan–Elson color reaction (Reissig *et al.*, 1955). [c] The enzyme activity units reported above were not the same as those used in the earlier work by Ghosh and Roseman (1964). It was observed several times in the present study that the specific activity of the starting material (*i.e.*, crude extract) was less active than that reported earlier. However, the specific activity of the purified enzyme in the present study was slightly higher than that of Ghosh and Roseman (1964) when calculated on the same basis. The enzyme activity reported above was calculated as μmoles of *N*-AcGm formed per min in contrast to earlier workers' report as μ moles of *N*-AcMm formed per 15-min incubation.

2 ml/min (100 ml each of the two respective elution media). Fractions were collected, 5 ml each, and assayed for ATP-dependent epimerase activity. The enzyme was detected between 0.09 and 0.11 M potassium phosphate buffer (pH 7.6), and these fractions were pooled (DEAE elute, 45 ml). The pooled fractions were dialyzed against 5 mM potassium phosphate buffer (pH 7.6) for 5 hr, and the dialyzed preparation was applied again to a DEAE-cellulose column (1 × 2 cm) equilibrated as before and the enzyme was eluted out with 3 ml of 0.2 M potassium phosphate buffer (pH 7.6). The concentrated enzyme fraction was again dialyzed against 5 mM potassium phosphate buffer (pH 7.6) for 12 hr and the dialyzed preparation was designated as "DEAE concentrate."

CALCIUM PHOSPHATE GEL. The DEAE-cellulose concentrate (3 ml) was treated with 35 mg of calcium phosphate gel (dry weight). After gentle stirring of the mixture for 10 min, the suspension was centrifuged. The supernatant fluid was designated as "calcium phosphate gel fraction."

A summary of the purification procedure is given in Table I. The procedure resulted in a purification of approximately 1500-fold with an overall recovery of about 37%.

Properties of 2-Epimerase. ATP-INDEPENDENT AND ATP-DEPENDENT ACTIVITIES OF VARIOUS ENZYME FRACTION. Previously, kinetic and other properties of the 2-epimerase were studied in the presence of ATP (Ghosh and Roseman, 1964). While present enzyme fractions were quite active in catalyzing the epimerization of *N*-AcMm to *N*-AcGm in the presence of ATP (Table I) it was of interest to examine the effect of exclusion of ATP from the incubation mixture. In this connection it should be mentioned that the epimerase preparation was free of ATP as tested according to the methods described in the text. As presented in Table I, the enzyme fractions that catalyzed the epimerization reaction in the presence of ATP, also catalyzed the same reaction in the absence of ATP, although at a much diminished rate in the latter case. As seen in Table I, the ratio of ATP-dependent and ATP-independent activities of the various enzyme fractions remained nearly constant (≃20) during entire purification procedure.

KINETIC PROPERTIES. The rate of ATP-independent enzymatic reaction was proportional to the protein concentration and was linear with time of incubation and the enzyme exhibited a pH optimum between 7.4 and 7.6 (Figures 1 and 2), similar to that observed by Ghosh and Roseman (1964) for ATP-dependent epimerase.

The effect of *N*-AcMm concentrations on the rate of ATP-

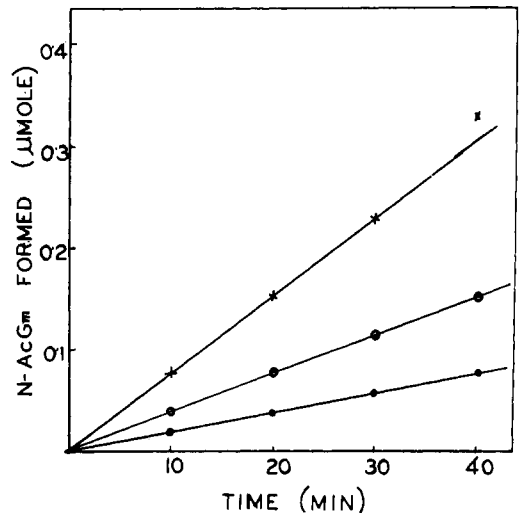

FIGURE 1: Effect of incubation time and protein concentration on the rate of ATP-independent epimerase reaction. The composition of incubation mixture and other assay conditions (except the period of incubation) were the same as described for measuring the ATP-independent activity of the epimerase under "Epimerase assay." The amount of enzyme protein added per tube was 10 μg (●—●) and 20 μg (○—○) from DEAE-cellulose fraction and 150 μg (×—×) from bentonite supernatant concentrate fraction.

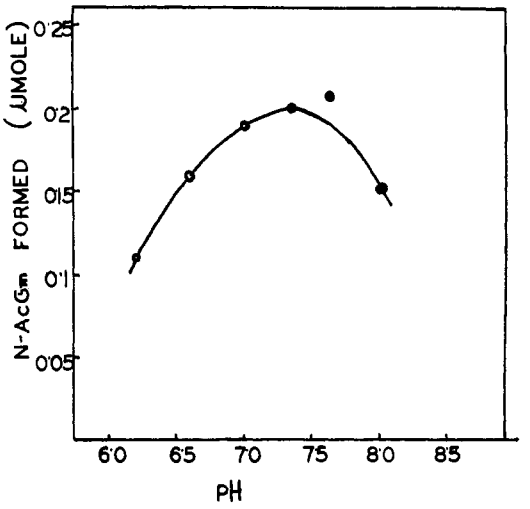

FIGURE 2: pH optima of the ATP-independent epimerase activity. Conditions of the assay and composition of the incubation mixtures were the same as described under "Epimerase assay" for ATP-independent epimerase activity, except that each incubation tube contained 12.5 μmoles of either potassium phosphate buffer (○) or Tris-HCl (●) and 150 μg of bentonite supernatant concentrate.

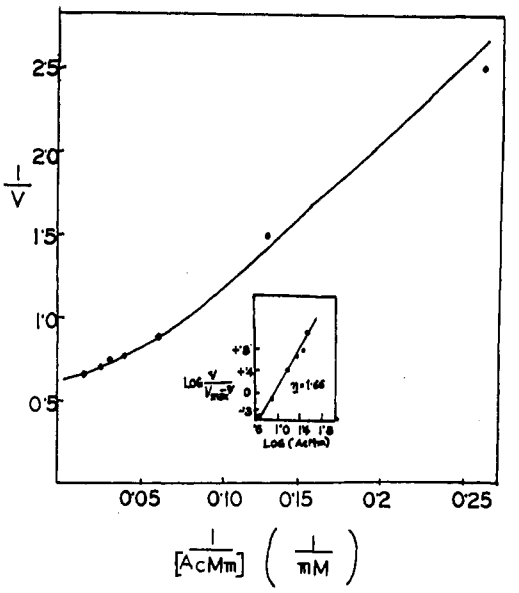

FIGURE 4: Double-reciprocal plots of ATP-independent reaction rate and *N*-AcMm concentration. The assay conditions were the same as described in Figure 3. The inset shows the above data plotted according to the empirical Hill equation; v, micromoles of *N*-AcGm formed per milliliter of assay mixture in 30 min.

independent epimerization reaction is shown in Figure 3. Apparent K_m value of *N*-AcMm (concentration of *N*-AcMm required for half-maximal velocity) calculated from Lineweaver–Burk plot was 9×10^{-3} M in the absence of ATP (Figure 4) whereas K_m value for *N*-AcMm in the presence of ATP was 1.7×10^{-3} M (Figure 5).

EFFECT OF ATP ON THE REACTION RATE. When the rate of epimerization was plotted against ATP concentration in the presence of excess *N*-AcMm, a typical sigmoid-shaped curve was obtained (Figure 6). *N*-AcMm concentration used in this study was high enough (4×10^{-2} M) for the enzyme to maintain high ATP-independent activity. As evident in Figure 6, ATP increased the V_{max} of the enzyme-catalyzed reaction by about 18-fold. Sigmoid shape of the ATP saturation curve is indicative of interactions between ATP binding sites.

For an enzyme possessing n number of mutually interacting substrate binding sites it has been shown by Atkinson

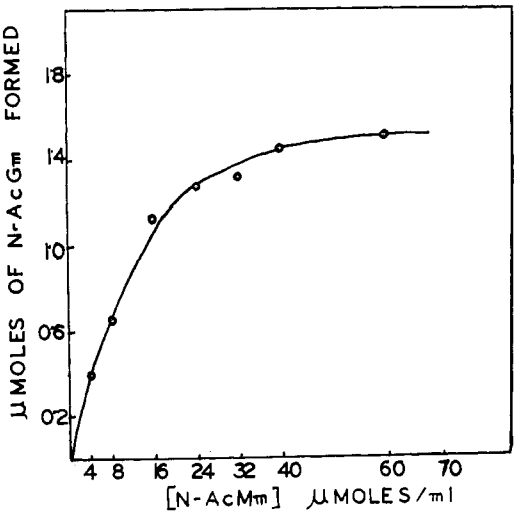

FIGURE 3: Effect of *N*-acetylmannosamine concentration on the ATP-independent reaction rate. The composition of incubation mixture and the other assay conditions were the same as described in the test for the assay of ATP-independent enzyme activity except for *N*-AcMm concentrate which was varied. Bentonite supernatant concentration fraction (340 μg of protein/assay mixture) was used.

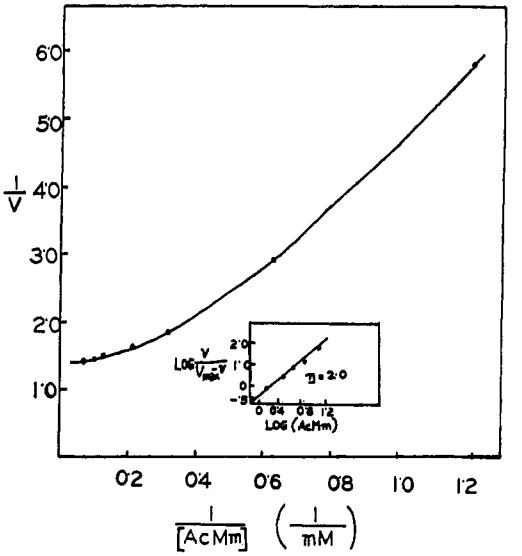

FIGURE 5: Double-reciprocal plots of reaction rate in the presence of ATP and *N*-AcMm concentration. The composition of incubation mixture and other assay conditions were the same as described in the text for the assay of ATP-dependent activity except for the varying concentration of *N*-AcMm. The inset shows the above data plotted according to the empirical Hill equation; v, micromoles of *N*-AcGm formed per milliliter of assay mixture in 30 min.

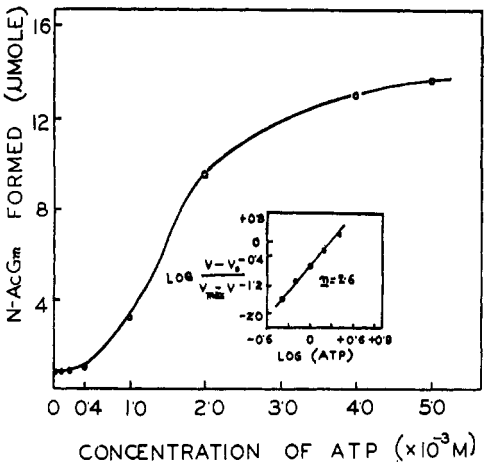

FIGURE 6: Activation of the 2-epimerase by ATP. Enzyme activity was plotted as a function of ATP concentration. The composition of incubation mixtures and other assay conditions were the same as described in the assay procedure of ATP-independent activity except for the varying concentrations of ATP; 30 μg of calcium phosphate gel fraction was used. The inset shows the data plotted in the form of Hill equation; v, micromoles of *N*-AcGm formed per milliliter of assay mixture in 10 min.

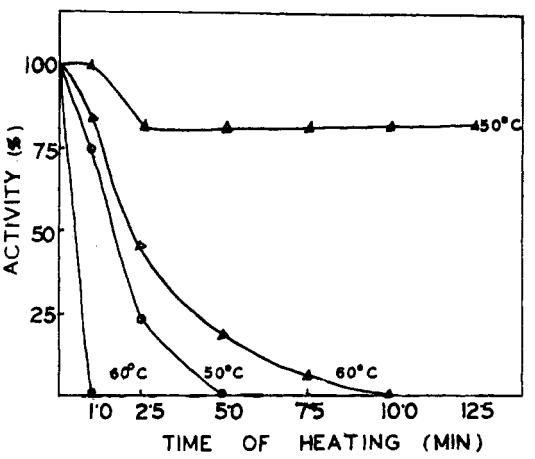

FIGURE 7: Effect of heat on sensitivity to ATP activation of *N*-Ac-Mm-2-epimerase. Bentonite supernatant, concentrated to 1.5 mg of protein/ml and dialyzed, was kept at 50 and 60° for the indicated time. The heat-treated enzyme fractions were assayed immediately for the ATP-dependent and ATP-independent epimerase activities using 15 μg and 150 μg of protein, respectively, by the procedure described in the text under "Epimerase assay." (O—O) ATP-dependent activity; (Δ—Δ) ATP-independent activity.

et al. (1965) that the Michaelis equation may be put in the form

$$\log \frac{v}{V_{max} - v} = n \log (S) - \log k \qquad (1)$$

where v is the velocity of the enzyme-catalyzed reaction, (S) is substrate concentration, V_{max} is the maximum velocity of the reaction, and k is constant. Equation 1, also known as Hill equation (Hill, 1913), has been widely used in the study of enzyme–substrate (or enzyme–effector) interactions in the case of regulatory enzymes which usually show sigmoidal response of activity to increasing substrate (or effector) concentrations.

For an enzyme where an effector enhances the rate of reaction as has been observed with *N*-AcGm–2-epimerase, one may show that the Hill equation may be transformed to

$$\log \frac{v - v_0}{V_{max} - v} = n \log (A) - \log k \qquad (2)$$

where v_0 = velocity of reaction in the absence of effector and (A) = concentration of effector. As presented in Figure 6, the plotting of log $[(v - v_0)/(V_{max} - v)]$ *vs.* log (ATP) gave a straight line whose slope, n (Hill coefficient or interaction coefficient), was equal to 2.6. According to Atkinson *et al.* (1965) the numerical value of n will depend both on the number of interacting sites and the strength of interactions between them. In cases where interaction is strong, the value of n approaches a limiting integral value equal to the number of sites. The numerical value of the slope, $n = 2.6$, in the present study suggests that there are at least three ATP binding sites per molecule of the 2-epimerase, assuming that the interaction was strong.

Densensitization of the Enzyme to ATP. As the site for an effector molecule on a regulatory enzyme is not identical with the site for its substrate, it has been possible, in many instances, to desensitize a regulatory enzyme to allosteric effector by structural modification of the protein without loss of its catalytic activity (Gerhart and Pardee, 1962; Martin, 1962; Banerde *et al.*, 1964). Among methods used to desensitize an enzyme to allosteric control, the treatment with PMB and heating are quite common. Both these methods were successfully used in the present study to desensitize the 2-epimerase to ATP requirement. (A) In an attempt to obtain the enzyme in a state quite insensitive to ATP requirement but retaining its ATP-independent catalytic function, bentonite supernatant fraction was subjected to various degrees of thermal denaturation treatment. As seen in Figure 7, the enzyme fraction

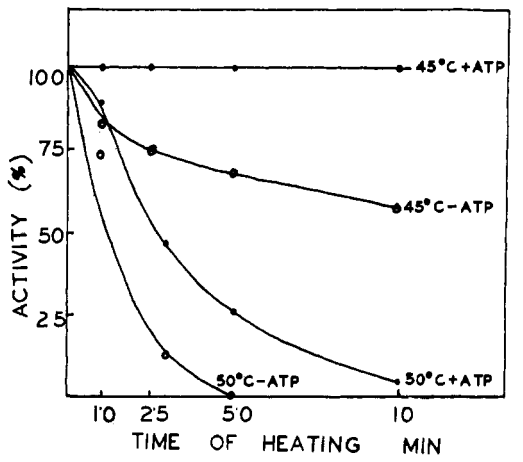

FIGURE 8: Protection by ATP against the thermal inactivation of the ATP binding site. The dialyzed bentonite supernatant concentrate, 1 5 mg of protein/ml, was kept at 45° and at 50° with 5 mM ATP and without ATP for the indicated time. The heat-treated enzyme fractions (aliquots containing 15 μg of protein) were assayed immediately for the ATP-dependent epimerase activities following the procedure in the text under "Epimerase assay."

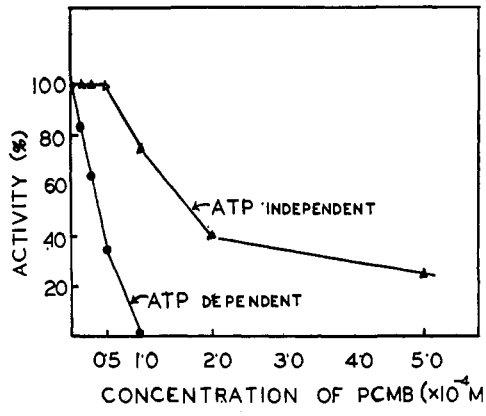

FIGURE 9: Effect of PMB on sensitivity to ATP activation of 2-epimerase. Calcium phosphate gel fraction (0.3 mg of protein/ml and dialyzed) was preincubated at 27° for 3 min with PMB at the given concentrations. The treated fractions were immediately diluted with the assay medium. The ATP-dependent and ATP-independent using 3 μg and 30 μg of protein, respectively, were assayed as described in the text under "Epimerase assay."

was fully desensitized to ATP by heating at 60° for 1 min whereas the same treatment resulted in a very slight loss of ATP-independent activity. At 50°, the desensitization was completed in 5 min but the desensitized enzyme retained about 80% of its initial ATP-independent activity even after 20-min heating. The most probable explanation of this differential effect is that the catalytic site and the site that binds ATP are not identical in nature.

Allosteric enzymes frequently become more resistant to heat denaturation by the presence of effector molecules (Martin and Vagelos, 1962). In fact, in the present study when ATP was present during the time of heating of the enzyme a protective effect against heat inactivation was observed (Figure 8). As evident in Figure 8, there was a distinct protective effect of ATP on the ATP-dependent activity during heating on the enzyme at 45° and at 50°.

(B) When the 2-epimerase was treated with PMB under controlled conditions, one can obtain the enzyme catalytically active but completely insensitive to ATP (Figure 9). The enzyme was found to be inactivated by PMB in two distinct steps. The first inactivation was accomplished by preincubating the enzyme with PMB at a concentration below 5×10^{-5} M. At or below 1×10^{-4} M, PMB was particularly effective in destroying the sensitivity of the enzyme to ATP (Figure 9). The second step which corresponded to the inactivation of the catalytic site, become operative when the concentration of PMB exceed 5×10^{-5} M (Figure 9). In addition the effect of PMB could be reversed in the presence of 2-mercaptoethanol (Table II) which suggests the possibility of the enzyme being to dissociated into subunits in the presence of PMB and being reassociated in the presence of 2-mercaptoethanol.

Substrate Saturation Curve of the Enzyme Desensitized to ATP. *N*-AcMm saturation curve of the native enzyme in the absence of ATP showed some deviation from Michaelis–Menten relationship as evident from its Lineweaver–Burk plot in Figure 4. The Lineweaver–Burk plot was slightly cruved instead of being linear. When the effect of increasing concentrations of *N*-AcMm on the enzyme activity was measured in the presence of ATP, a more pronounced deviation from Michaelis–Menten relationship was observed (Figure 5). The substrate saturation curves, in the absence and in the presence of ATP were plotted in the Hill system of coordinates and are shown in Figures 4 and 5, respectively. It is apparent that the presence of ATP has induced a change in the slope of the Hill plot from 1.66 to 2.0. On the other hand, the substrate saturation curves of the desensitized enzyme obtained by PMB treatment or by heating conform to the Michaelis–Menten kinetics as is seen from the linear form of their Lineweaver–Burk plot (Figures 10 and 11). As expected, Hill plots of these curves have yielded straight lines having their slope close to 1.

TABLE II: Reversal of PMB Inhibition of ATP-Dependent Activity by 2-Mercaptoethanol.[a]

PMB Concn (M)	*N*-AcGm Formed (μmole/assay tube)	
	Without 2-Mercaptoethanol	With 2-Mercaptoethanol
0	0.24	0.24
0.5×10^{-4}	0	0.24
1.0×10^{-4}	0	0.20
2.0×10^{-4}	0	0.125

[a] Bentonite supernatant (concentrated to 1.5 mg of protein/ml and dialyzed) was preincubated at 27° for 3 min with PMB at the given concentrations. The treated fraction was immediately diluted with the assy medium for ATP-dependent activity measurement. The composition of incubation mixtures and the other conditions were the same as described in the text for measuring ATP-dependent epimerase activity except that 2-mercaptoethanol was added (final concentration 2×10^{-2} M) in each of the duplicate tubes.

Discussion

It was shown that ATP was nonessential in the reaction catalyzed by 2-epimerase by using high enzyme and high substrate concentrations (Table I). The ATP-dependent and ATP-independent empimerase activities were not separated during the course of purification procedure and the ratio of the specific activities remained essentially constant throughout the 1500-fold purification of the enzyme. It therefore appears that the same enzyme was responsible for both activities.

Allosteric enzymes whose activities are dependent on the presence of their effector molecules, though rare, have been reported in literature. The best-known examples are acetyl-CoA carboxylase which requires citrate (Martin and Vagelos, 1962) and pyruvate carboxylase which requires acetyl-CoA (Scrutton and Utter, 1965) for their activities. In addition, the activation of frog muscle glycogen synthetase D requires glucose 6-phophate (Rosell-Perez and Larner, 1962). Ghosh and Roseman (1964) concluded that *N*-AcGm-2-epimerase was absolutely dependent on ATP for its catalytic activity. However, the present study has demonstrated apparent nonessentiality of ATP for the activity of 2-epimerase, though

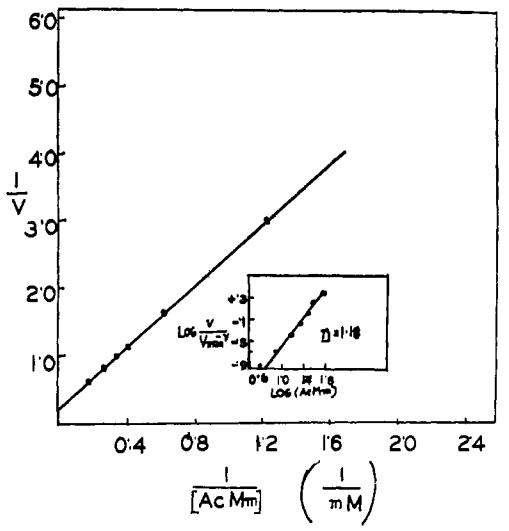

FIGURE 10: Lineweaver–Burk plot of reaction rate of heated enzyme to increasing concentrations of *N*-AcMm. The composition of incubation mixtures was the same as described in the text for the assay of ATP-independent enzyme activity except for the *N*-AcMm concentration which was varied; 340 μg of protein from the desensitized enzyme preparation (heated 50° for 5 min, as described in Figure 7) was used per assay tube. The inset shows the data plotted according to the empirical Hill equation; v, micromoles of *N*-AcGm formed per milliliter of assay mixture in 30 min.

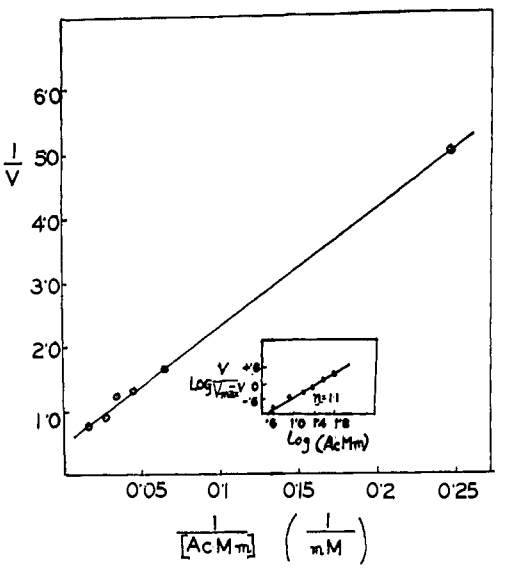

FIGURE 11: Lineweaver–Burk plot of reaction rate of PMB-treated enzyme to increasing concentrations of *N*-AcMm. Bentonite supernatant concentrate was preincubated at 27° for 3 min with 5×10^{-5} M PMB and then dialyzed. The treated fraction (340 μg of protein) was immediately diluted with the assay medium and assayed as described in Figure 10.

its presence increased the V_{max} by a factor of 20. When the activator of an enzyme enhances the V_{max} by this order of magnitude, it is not unlikely that the enzyme is catalytically inactive in the absence of its allosteric effector (especially when the sensitivity of the assay method is not very high).

Because of ATP's remarkable effect on V_{max} in the positive direction, 2-epimerase appears to belong to a group of allosteric enzymes of the type known as positive "V system," as defined by Monod *et al.* (1963). Furthermore, the activation of 2-epimerase by ATP was accompanied by an increase of the enzyme's affinity for its substrate, *N*-AcMm (Figure 3). Figures 3 and 6 illustrates the effect of *N*-AcMm and ATP concentrations on the enzyme activity. A hyperbolic relationship was noted in the case of *N*-AcMm (Figure 3), while in case of ATP (Figure 6) sigmoidal relationship was seen. When the data of Figure 3 were plotted in terms of $1/v$ with respect to 1/(S), a straight line was not obtained (Figure 4). The relationship of these two reciprocal entities was a curve. This type of nonlinear relationship is indicative of the presence of separate binding sites for activator and substrate molecules, and this has been interpreted as an indication of enzyme kinetics of "allosteric" type (Morrison, 1965; Cleland, 1963; Sanwal *et al.*, 1966).

As noted by a number of authors (Monod *et al.*, 1963; Atkinson *et al.*, 1965; Rubin and Changeux, 1966) the Hill plot (Hill, 1913) is a useful tool for analyzing some allosteric effects, and the values of the slopes obtained are considered to be a measure of the number of interacting sites as well as the strength of their interaction. When the interaction is very strong, the value of the slope of the Hill plot approaches the number of binding sites for the ligand. The kinetic data of 2-epimerase action with respect to substrate were fitted to the empirical Hill equation. By plotting $\log v/(V_{max} - v)$ against log (S) as shown in Figure 4, a straight line was obtained whose slope was equal to 1.66. This value implies that the number of "interacting active sites" is at least 2. When the Hill equation was applied to the kinetics of activation of ATP, the value of n became 2.6 (Figure 6) suggesting that at least three binding receptor sites for ATP might exist per molecule of the enzyme.

The first indication that the sites for the substrate molecule and for the effector molecule were distinct occurred after treatments which abolished the sensitivity of the enzyme to ATP and simultaneously normalized the kinetics of *N*-AcMm epimerization.

When the specific activating effect of ATP on the 2-epimerase was destroyed by heating or PMB treatment of the enzyme, a concomitant abolition of the cooperativity of substrate binding and an enhancement of the apparent K_m value observed (Table III). PMB or heat treatment might have weakened or damaged the quaternary bonds leading to a simultaneous loss of the homotropic interactions (of substrate ligands) and the heterotropic interactions without destroying the ATP-independent catalytic activity of the 2-epimerase. On the basis of the Monod's model (Monod *et al.*, 1965), conservation of the interactions should depend on the integrity of the whole native structure, including in particular the interprotomer binding, whereas conservation of the activity should only depend on the integrity of the active site. According to the model, loss of the interactions observed in the present study (Table III) may follow from any structural alteration of protein.

A distinctive kinetic feature of the 2-epimerase is the inverse relationship between the cooperativity of substrate binding (Hill coefficient) and the apparent K_m value for the substrate. As can be seen from Table III any process that weakens the substrate interaction also weakens the substrate affinity (*i.e.*, increases the concentration of substrate required for the

TABLE III: Hill Coefficients and Apparent K_m Values with Respect to *N*-AcMm of the 2-Epimerase.

Enzyme Fraction	Addition	App K_m (Concn of *N*-AcMm at $^1/_2$ V_{max}) $\times 10^{-3}$ M	Hill Coeff (n)
Bentonite supernatant concentrate	ATP	1.7	1.94
Bentonite supernatant concentrate		9.0	1.66
Bentonite supernatant concentrate desensitized by heat		29.0	1.2
Bentonite supernatant concentrate desensitized by PMB		38.0	1.1

half-maximal velocity) for the enzyme. The conformation of the 2-epimerase in the active state not only promotes cooperativity of substrate binding but it also leads to a greater affinity of the enzyme for its substrate. In other words, "conformational constrains" acting on the "promoters" is a necessary condition for higher catalytic activity and higher substrate affinity in the case of the allosteric protein, *N*-AcGm–2-epimerase.

Regulatory significance of the sensitivity of 2-epimerase to ATP is not very clear. 2-Epimerase is the first enzyme in the pathway leading to the formation of CMP-sialic acid: *N*-AcGm ↔ *N*-AcMm → *N*-AcMm-6-P → sialic acid-9-P → sialic acid → CMP-sialic acid (Ginsburg, 1964; Kornfeld *et al.*, 1964). CMP-sialic acid is a precursor for the biosynthesis of glycoproteins and mucopolysaccharides. These macromolecules, along with proteins, are extensively utilized in the building up of connective tissues, blood cells, and other structural elements in mammalian systems. From the point of view of metabolic control, 2-epimerase seems to occupy a strategic position in the metabolism of amino sugars. Since the reversible reaction catalyzed by 2-epimerase is biologically important for the synthesis of *N*-AcMm, which is necessary for the biosynthesis of CMP-sialic acid and since 2-epimerase is found in many animal tissues, including those that actively produce sialic acid polymers, one may think that any significant formation of sialic acid and sialic acid polymers would depend on and will be accelerated by an increased activity of 2-epimerase, which for its maximal activity, requires elevated concentrations of ATP inside a cell. In other words, the biosynthesis of structural macromolecules containing sialic acid will be adversely affected by low ATP concentrations. Further work is now in progress to study the regulatory significance of ATP in this and other subsequent steps of sialic acid biosynthesis.

Acknowledgments

The author expresses his profound gratitude to Dr. S. Ghosh for his constant guidance and advice and for providing laboratory facilities. The author thankfully acknowledges the valuable suggestions of Professors D. M. Bose and A. Sen during this investigation. He also thanks Dr. R. K. Datta of Beth Israel Medical Center, New York, Miss Pamela Wolfe of The Public Health Research Instutue, New York, and Dr. K. Datta of New York Research Institute for Neurochemistry and Drug Addiction for their kind help in preparing the manuscript.

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