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PURIFICATION AND PROPERTIES OF UDP-*N*-ACETYLGLUCOSAMINE 2′-EPIMERASE FROM RAT LIVER

KUNIMI KIKUCHI AND SHIGERU TSUIKI

Biochemistry Division, Research Institute for Tuberculosis, Leprosy and Cancer, Tohoku University, Sendai (Japan)

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SUMMARY

1. UDP-*N*-acetylglucosamine 2′-epimerase (EC 5.1.3.7) has been purified 500-fold from rat liver with UDP as stabilizing agent. In the presence of UDP and dithiothreitol, the final preparation is stable and loses only 20% of the initial activity over 3 days storage at 4 °C.

2. UDP and UDP-*N*-acetylglucosamine protect 2′-epimerase from inactivation by aging. UDP also is a competitive inhibitor, the K_i being 0.14 mM. Uridine, the stabilizing agent used by previous workers, neither stabilized nor inhibited the enzyme. It is suggested that UDP interacts with the enzyme at the substrate site. Dithiothreitol also stabilizes the enzyme but only under limited conditions.

3. 2′-Epimerase exhibits negative cooperativity in UDP-*N*-acetylglucosamine binding. The apparent K_m value in the lower activity range is 0.08 mM. The negative cooperativity is abolished by UDP or CMP-*N*-acetylneuraminic acid.

4. 2′-Epimerase is highly sensitive to inhibition by CMP-*N*-acetylneuraminic acid; the concentration required for 50% inhibition (0.025 mM) is close to its intracellular levels. A Hill plot yielded the Hill coefficient (n_H) of 5.7.

5. When UDP but not dithiothreitol is present, the purified enzyme undergoes alterations in UDP-*N*-acetylglucosamine binding such as the disappearance of negative cooperativity, an increase in K_m and the appearance of substrate inhibition; all are reversed by dithiothreitol.

INTRODUCTION

UDP-*N*-acetylglucosamine (UDP-GlcNAc) 2′-epimerase (EC 5.1.3.7) catalyzes the formation of *N*-acetylmannosamine from UDP-GlcNAc[1] and is subject to feedback inhibition by CMP-*N*-acetylneuraminic acid (CMP-NANA)[2]. We have recently reported that 2′-epimerase activity in rat liver rises along with development and

Abbreviations: UDP-GlcNAc, UDP-*N*-acetylglucosamine; CMP-NANA, CMP-*N*-acetylneuraminic acid; DTT, dithiothreitol.

falls upon cancerization[3]. These together suggest that the enzyme is at a key site of control of synthesis of CMP-NANA and, hence, of glycoproteins.

Although several useful studies have been made of 2′-epimerase of rat liver[2,4–7], the enzyme preparations were extremely unstable and the kinetic data were largely inconsistent. In the present study, we have prepared the enzyme from rat liver in a highly purified and stable state. The kinetic properties of the purified enzyme differ from those reported previously[2,4–7] in such a way that secondary modifications of the enzyme are minimum, if any, under our experimental conditions.

MATERIALS AND METHODS

Crude enzyme preparations

Step 2 enzyme. 5 normal well-fed rats of the Donryu strain were killed and their livers were quickly removed, and rinsed. Subsequent operations were performed at 0–4 °C. The livers were homogenized for 1 min in a glass-Teflon homogeneizer using 2 vol. of 0.154 M KCl containing 0.15 mM UDP. The homogenate was centrifuged for 1 h at 105 000 × *g* and the supernatant (the step 1 enzyme) was brought to 40% saturation with saturated $(NH_4)_2SO_4$ solution. Following centrifugation for 10 min at 18 000 × *g*, the precipitate was dissolved in 5 ml of 20 mM sodium phosphate (pH 7.5) and passed through a desalting column (1.5 cm × 50 cm) of Sephadex G-25. The protein eluates are called the step 2 enzyme.

Step 3′ enzyme. The $(NH_4)_2SO_4$ precipitate prepared as above (120 mg in protein) was dissolved in a minimum volume of 20 mM sodium phosphate (pH 7.5)–40 mM KCl–0.1 mM UDP and $(NH_4)_2SO_4$ was removed by gel filtration. The protein eluates were combined (5 ml) and applied to a column (1.5 cm × 7 cm) of TEAE-cellulose previously equilibrated with 20 mM sodium phosphate (pH 7.5)–40 mM KCl–0.1 mM UDP. The column was washed with 30 ml of equilibrating buffer and 2′-epimerase activity was eluted from the column with 20 mM sodium phosphate (pH 7.5)–0.1 M KCl–0.1 mM UDP. The enzyme eluates (3 ml) were then applied to a column (1.5 cm × 20 cm) of Sephadex G-25 and eluted with 20 mM sodium phosphate (pH 7.5). The eluates are called the step 3′ enzyme.

Assay for 2′-epimerase

Preliminary studies revealed that phosphate can stabilize 2′-epimerase to a certain extent while Tris is toxic to the enzyme. The incubation mixture in a final volume of 1 ml thus contained the following components in μmoles: sodium phosphate (pH 7.5), 45; UDP-GlcNAc, 1; $MgCl_2$, 10; EDTA, 0.01; dithiothreitol, 1; UDP, 0.02; and enzyme. After 20 min at 37 °C, the mixtures were heated for 2 min at 100 °C, centrifuged and the supernatants were assayed for *N*-acetylmannosamine by the method of Reissig *et al.*[8] as modified by Spivak and Roseman[9]. A unit of enzyme was defined as the amount which catalyzed the formation of 1 nmole of *N*-acetylmannosamine per h. Protein concentrations were measured using a phenol reagent[10].

Preparation of CMP-NANA

CMP-NANA synthetase was prepared by homogenizing rat brain (12.9 g) with 2 vol. of 0.154 M KCl–1 mM EDTA followed by centrifugation for 1 h at 105 000 × *g*

and fractionation with $(NH_4)_2SO_4$. The precipitate formed between 40 and 60% saturation was dissolved in 2 ml of 10 mM Tris–HCl (pH 7.6) and after dialysis for 1 h against the same buffer, the solution was incubated for 3 h at 37 °C with 20, 20, 25 and 108 μmoles of NANA, CTP, $MgCl_2$ and Tris–HCl (pH 9.0), respectively, in a final volume of 5 ml. The mixture was then chilled, centrifuged and the supernatant was deproteinized by passing through a Sephadex G-25 column. The isolation of CMP-NANA was conducted according to Kean and Roseman[11]. The yield was 4.2 μmoles as determined by the thiobarbituric acid method of Warren[12].

Chemicals

The following materials were obtained from commercial source: UDP-GlcNAc, uridine, UMP, UDP, UTP and CTP from Boehringer; and NANA and crystalline bovine serum albumin from Sigma. Hydroxyapatite was purchased from Seikagaku Kogyo Co., Tokyo.

RESULTS

Protection from inactivation

Previous workers[4–6] reported that partially purified 2′-epimerase is extremely unstable and loses all activity within a few hours. In agreement with these reports, both the step 2 and step 3′ enzymes were rapidly inactivated by incubation at pH 7.5 at 4 °C (Fig. 1). If incubation was carried out at 37 °C, the half-life of the step 2

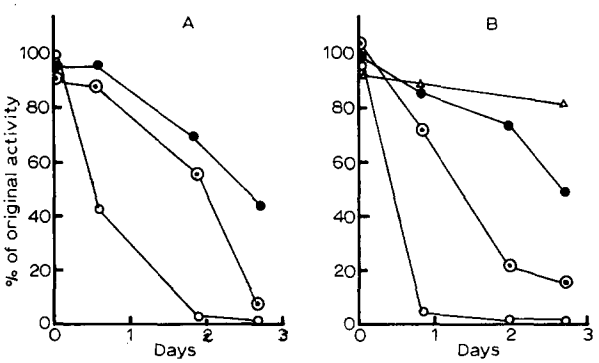

Fig. 1. Inactivation of 2′-epimerase at 4 °C and protecting effect of UDP and DTT. The step 2 enzyme (A) or step 3′ enzyme (B) in 20 mM sodium phosphate (pH 7.5) was incubated at 4 °C in the presence of none (○), 0.1 mM UDP (●), 1 mM DTT (○), or 0.1 mM UDP *plus* 1 mM DTT (△).

enzyme was only about 2 min (Fig. 2). The inactivation is due to a reduction in V (rather than a rise in K_m) and was markedly prevented by 0.1 mM UDP (Figs 1 and 2). UDP, however, did not restore the activity of 2′-epimerase that had been inactivated in a prior incubation. Therefore, the inactivation is essentially irreversible.

Spivak and Roseman[4] reported that uridine, UDP or various analogues of these compounds (but not UMP) can stabilize the enzyme; all previous purification studies[4–7] were, therefore, conducted in the presence of 0.5 mM uridine. Table I,

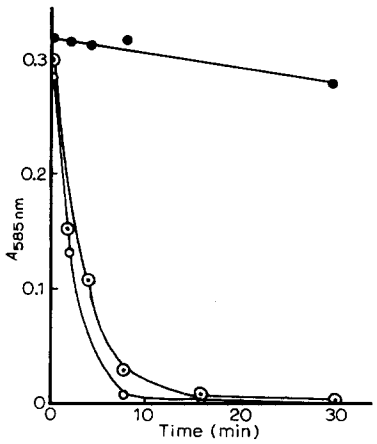

Fig. 2. Inactivation of 2′-epimerase at 37 °C and protecting effect of UDP. The step 2 enzyme in 20 mM sodium phosphate (pH 7.5) was incubated at 37 °C in the presence of none (○), 0.1 mM UDP (●), or 1 mM DTT (○).

however, reveals that only UDP and UDP-GlcNAc, and not uridine, can stabilize the step 3′ enzyme at 4 °C. Half-maximal stabilization was attained by 0.024 mM UDP and 0.020 mM UDP-GlcNAc (Fig. 3). It should be noted that in these stability studies, no UDP was formed from UDP-GlcNAc.

TABLE I

EFFECT OF UDP AND RELATED COMPOUNDS ON INACTIVATION OF UDP-*N*-ACETYLGLUCOSAMINE 2′-EPIMERASE AT 4 °C

The step 3′ enzyme in 20 mM sodium phosphate (pH 7.5) was incubated at 4 °C for 20 h in the presence of the compounds listed below. The final concentration of the additions was 0.36 mM.

Compounds	*Residual activity (%)*
AMP	0
ADP	0
ATP	0
Uracil	0
Deoxyuridine	0
Uridine	2
UMP	2
UDP	85
UTP	24
UDP-glucose	2
UDP-*N*-acetylglucosamine	89

As shown in Fig. 1A, DTT was almost as effective as UDP in protecting the step 2 enzyme from inactivation at 4 °C. Albumin and EDTA were without effect. Rapid and irreversible inactivation brought about by *N*-ethylmaleimide at 4 °C, on the other hand, was markedly prevented by UDP (Fig. 4). While these observations may suggest that certain–SH groups of 2′-epimerase play a role in conformational stability at low temperature, DTT failed to stabilize the same enzyme preparation

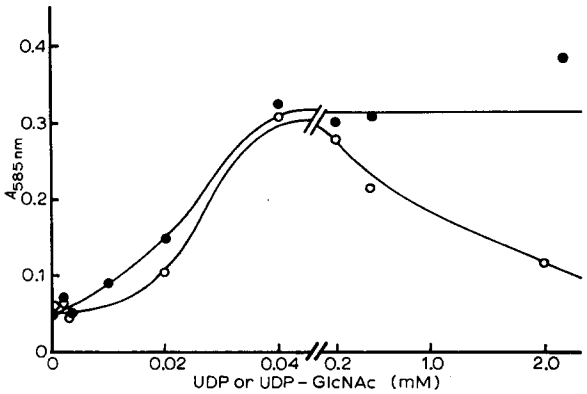

Fig. 3. Stability of 2′-epimerase as a function of UDP or UDP-GlcNAc concentration. The step 3′ enzyme in 20 mM sodium phosphate (pH 7.5) was incubated at 4 °C for 20 h in the presence of varying concentrations of UDP (○), or UDP-GlcNAc (●). When the concentration of UDP was greater than 0.2 mM, the amounts of UDP sufficient to inhibit 2′-epimerase (see text) were carried over to the assay mixture.

at 37 °C (Fig. 2). In addition, the stabilization of 2′-epimerase by DTT at 4 °C becomes progressively less marked as the enzyme is purified (Fig. 1 and see below).

Purification

All steps of the purification were performed at 0–4 °C and in the presence of 0.1 mM UDP. Under these conditions, the stability of 2′-epimerase was not particularly enhanced by the presence of *N*-acetylglucosamine, *N*-acetylmannosamine, NAD, UDP-GlcNAc, DTT or EDTA. All centrifugations were conducted at 18 000 × *g* for 10 min.

In preliminary experiments, the $(NH_4)_2SO_4$ precipitate was fractionated by

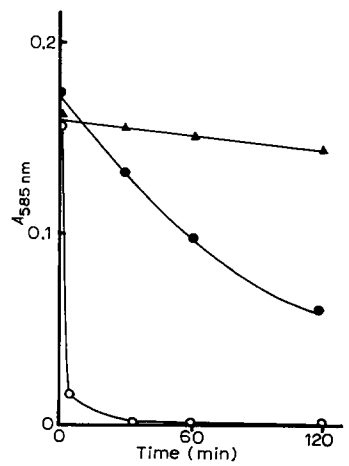

Fig. 4. Inactivation of 2′-epimerase by *N*-ethylmaleimide and its prevention by UDP. The step 2 enzyme in 20 mM sodium phosphate (pH 7.5) was incubated at 4 °C in the presence of none (●), 0.0075 mM *N*-ethylmaleimide (○), or 0.0075 mM *N*-ethylmaleimide *plus* 0.5 mM UDP (▲). Aliquots were removed and assayed in the presence of 0.003 mM *N*-ethylmaleimide and 0.2 mM UDP.

columns of DEAE-cellulose, TEAE-cellulose, DEAE-Sephadex or hydroxyapatite with gradient elution technique. 2′-Epimerase activity emerged always as a single peak. Since, however, recovery was extremely poor even in the presence of UDP and DTT, the experiments described below employed stepwise elution from short (1.0 cm or 0.5 cm) columns in order to complete fractionation as quickly as possible and this was essential for the optimum results.

Steps 1 and 2. Extraction (step 1) and $(NH_4)_2SO_4$ fractionation (step 2) were already described in Materials and Methods. Freshly prepared $(NH_4)_2SO_4$ precipitate was dissolved in 5 ml of 40 mM potassium phosphate (pH 7.0) containing 0.1 mM UDP and passed through a desalting column (1.5 cm × 50 cm) of Sephadex G-25.

Step 3: Protamine sulfate fractionation. To 14 ml of the combined protein eluates from above, protamine sulfate was added to a final concentration of 0.1%. After 10 min, the precipitate was collected by centrifugation and dissolved in 10 ml of 0.1 M potassium phosphate (pH 7.0) containing 0.1 mM UDP. The resulting solution was centrifuged.

Step 4: the second $(NH_4)_2SO_4$ fractionation. The above supernatant was brought to 30% saturation with saturated $(NH_4)_2SO_4$ solution containing 0.1 mM UDP and after centrifugation, the supernatant was brought to 40% saturation. The precipitate formed was collected by centrifugation, dissolved in 0.8 ml of 10 mM potassium phosphate (pH 7.0)–1 mM EDTA–0.1 mM UDP and passed through a desalting column (1.5 cm × 10 cm) of Sephadex G-25.

Step 5: hydroxyapatite fractionation. The protein eluates from above, 5 ml, were applied to a column (1.5 cm × 1.0 cm) of hydroxyapatite previously equilibrated with 10 mM potassium phosphate (pH 7.0)–1 mM EDTA–0.1 mM UDP. The column was washed with 20 ml of 50 mM potassium phosphate (pH 7.0)–1 mM EDTA–0.1 mM UDP and 2′-epimerase was eluted with 0.1 M potassium phosphate (pH 7.0)–1 mM EDTA–0.1 mM UDP.

Step 6: TEAE-cellulose fractionation. The active fractions from the hydroxyapatite column (9 ml) were diluted with 2 vol. of distilled water containing 0.1 mM UDP and applied to a column (1.5 cm × 0.5 cm) of TEAE-cellulose previously equilibrated with 20 mM sodium phosphate (pH 7.5)–0.1 mM UDP–40 mM KCl. The column was washed with 20 ml of the same buffer and 2′-epimerase was eluted with 20 mM sodium phosphate (pH 7.5)–0.1 mM UDP–0.1 M KCl.

The procedure described here consistently yielded about 500-fold purification and 5–10% yield. Table II contains data for a typical experiment. The final fraction is called the step 6 enzyme.

Stability and general properties

At pH 7.5, the step 6 enzyme was readily and irreversibly inactivated if UDP was absent. In the presence of 0.1 mM UDP and 1 mM DTT, however, the enzyme was stable and lost only 20% of its activity over 3 days storage at 4 °C. Under the standard assay conditions, the progress curve of enzyme reaction was linear; inhibition by UDP described below was not apparent unless incubation time exceeded 60 min.

In confirmation of the results of previous authors[6], a molecular weight of 300 000 to 400 000 was estimated from gel filtration with Sephadex G-200. In 50 mM Tris–maleate buffer, the step 6 enzyme showed maximal activity between pH 8.0

TABLE II

PURIFICATION OF UDP-*N*-ACETYLGLUCOSAMINE 2′-EPIMERASE FROM RAT LIVER

Steps	*Fractions*	*Total protein (mg)*	*Specific activity (units/mg)*	*Purification (fold)*	*Recovery (%)*
1	105 000 × *g* supernatant	1 398	120	(1)	(100)
2	The first $(NH_4)_2SO_4$ precipitate	227	540	4·5	73
3	Protamine fractionation				
4	The second $(NH_4)_2SO_4$ precipitate	11.3	3 672	31	25
5	Eluates from hydroxyapatite column	1.95	10 602	88	12
6	Eluates from TEAE-cellulose column	0.19	59 664	497	7

and 8.5 with an additional peak between pH 7.0 and 7.5. The step 6 enzyme was readily and irreversibly inactivated by –SH reagents such as *p*-chloromercuribenzoate, *N*-ethylmaleimide and iodoacetamide at 37 °C (Fig. 5). The data also demonstrates that as the enzyme is purified, it becomes progressively more susceptible to these reagents.

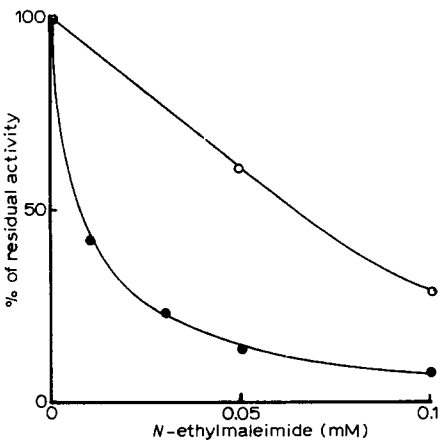

Fig. 5. Effect of purification on susceptibility of 2′-epimerase to *N*-ethylmaleimide. The step 2 enzyme (○), or step 6 enzyme (●) was assayed under the standard conditions in the presence of indicated concentrations of *N*-ethylmaleimide.

Effect of substrate concentration

When enzyme velocities were measured and plotted as a function of UDP-GlcNAc concentration, an upward deviation from the Michaelis–Menten curve was noted at concentrations greater than 0.5 mM (Fig. 6A). The R_8 value[13] derived from these data was 162, greater than the value of 81 which is expected for Michaelis–Menten curves. A double-reciprocal plot yielded two straight lines with different slopes, which extrapolated to apparent K_m values of 0.08 and 0.29 mM, respectively (Fig. 6B). Such a break in a double-reciprocal plot has not been demonstrated previously; the previous literature only gives a single K_m value of 0.3 (ref. 2), 2 (ref. 4) or 0.19 mM (ref. 6). A Hill plot of the velocity data gave a n_H value smaller than unity.

The anomalous kinetics was also observed with the step 2 enzyme: the inflection

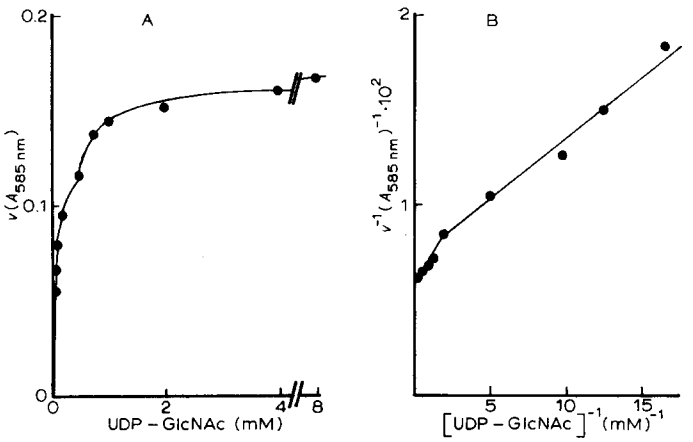

Fig. 6. Effect of UDP-GlcNAc concentration on velocity of 2′-epimerase reaction (the step 6 enzyme). A: UDP-GlcNAc saturation curve. B: Lineweaver–Burk plot.

point of saturation curve (0.5 mM) and the R_8 value (171) were the same as those for the step 6 enzyme. It is concluded that the anomalous kinetics described here is due to one enzyme undergoing negative cooperative interaction, as defined by Conway and Koshland[14], rather than the combined action of two Michaelis–Menten isoenzymes.

Inhibition by UDP

UDP inhibited 2′-epimerase as has been reported by Sommar and Ellis[7]. They, however, reported that the enzyme was inhibited in a linear non-competitive manner.

As shown in Fig. 7A, addition of UDP released the enzyme from negative

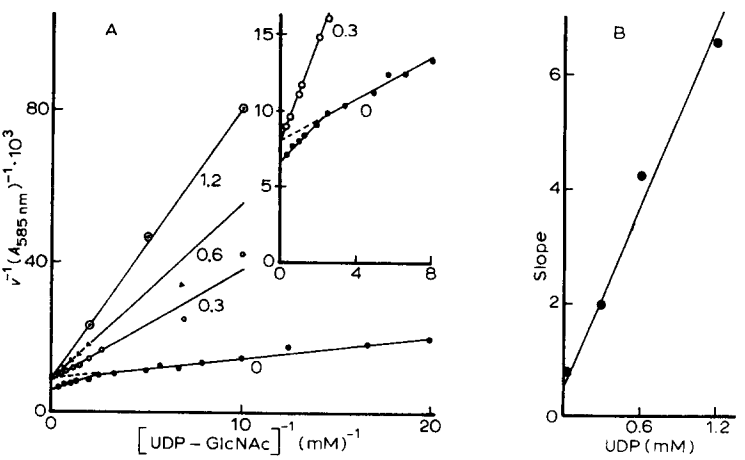

Fig. 7. Inhibition of 2′-epimerase by UDP. The step 6 enzyme was assayed with varying concentrations of UDP-GlcNAc at several fixed UDP concentrations. A: Lineweaver–Burk plot; the insert shows the data at the higher UDP-GlcNAc concentrations on an expanded scale. Indicated are the concentrations of UDP added exogenously and note that the standard assay mixture already contained 0.02 mM UDP as given in text. B: plot of the slopes as a function of UDP concentration.

cooperative interaction; a single straight line intersected the ordinate axis at the same intercept was obtained with lower concentrations of substrate in the absence of UDP. Furthermore, raising UDP concentration did not alter the V but raised the K_m for UDP-GlcNAc.

It is evident from these observations that UDP is an essentially competitive inhibitor with UDP-GlcNAc. Replotting the slopes of lines in Fig. 7A against UDP concentration yielded a linear line and a K_i value of 0.14 mM (Fig. 7B).

At a concentration of 4 mM, UDP, uridine, UMP, UTP and UDP glucose inhibited 2′-epimerase by 70, 2. 8, 39 and 37%, respectively. The degree of inhibition is in parallel with the capacity to stabilize the enzyme except for UDP glucose (Table I), suggesting that UDP and UDP-GlcNAc protect the enzyme chiefly by their ability to combine with the substrate site.

Inhibition by CMP-NANA

The effect of varying concentrations of CMP-NANA on 2′-epimerase activity is shown in Fig. 8A. In agreement with previous reports[2,6], the inhibition curve is sigmoid. But a Hill plot (Fig. 8B) yielded a value for n_H of 5.7 instead of 4 as reported by Sommar and Ellis[6]. Thus the positive homotropic cooperativity in CMP-NANA binding is more extensive with the present than with previous preparations.

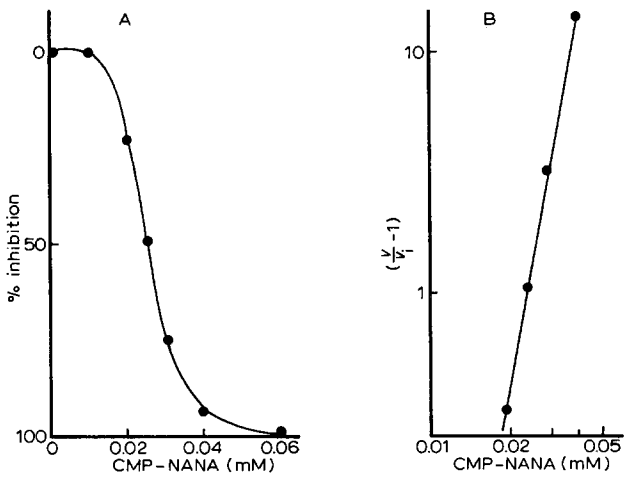

Fig. 8. Inhibition of 2′-epimerase by increasing concentrations of CMP-NANA (the step 6 enzyme). A: the inhibition curve. B: the Hill plot.

The present enzyme is also more susceptible to CMP-NANA inhibition than are the previous preparations. As can be seen in Fig. 8A, half-maximal inhibition is attained at a CMP-NANA concentration of 0.025 mM. The value is about an order of magnitude lower than those reported by previous authors[2,6] and almost equal to the level of CMP-NANA in rat liver[15]. Furthermore, a complete inhibition can be attained at inhibitor concentrations greater than 0.06 mM.

Fig. 9 shows that CMP-NANA alters both the V and K_m of 2′-epimerase. It should be pointed out that CMP-NANA, like UDP, abolishes negative cooperativity in the UDP-GlcNAc binding of 2′-epimerase.

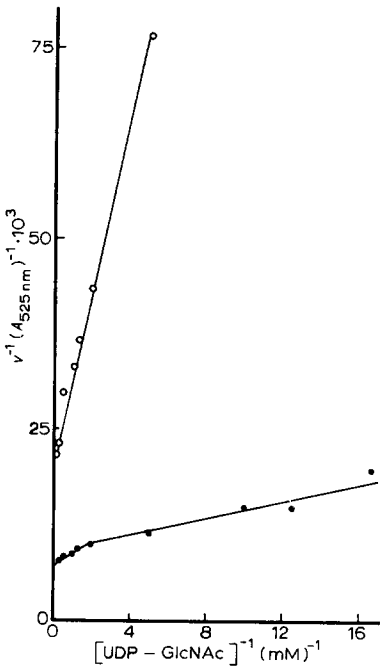

Fig. 9. Effect of UDP-GlcNAc concentration on inhibition of 2′-epimerase by CMP-NANA. The step 6 enzyme was assayed with varying concentrations of UDP-GlcNAc in the absence (●), or presence (○) of 0.03 mM CMP-NANA.

Effect of aging

When the step 6 enzyme was incubated at pH 7.5 at 4 °C in the presence of UDP but in the absence of DTT, the enzyme activity was lost gradually. This inactivation, however, was readily reversed by addition of DTT. The results of a typical experiment are shown in Table III.

The extent to which the enzyme undergoes this reversible inactivation varies considerably with different batches of the enzyme. The reason for this variation is

TABLE III

EFFECTS OF DITHIOTHREITOL, EDTA AND ALBUMIN ON INACTIVATION OF STEP 6 ENZYME AT 4 °C

The step 6 enzyme in 20 mM sodium phosphate (pH 7.5)–0.1 mM UDP–0.1 M KCl was incubated at 4 °C for 24 h in the presence of the compounds listed below. Assays were then made in the absence or presence of DTT.

Aging (h)	*Additions*		*Activity (%)*
	At aging	*At assay*	
0		none	100
0		DTT (1 mM)	111
24	none	none	7
24	none	DTT (1 mM)	90
24	DTT (1 mM)	none	103
24	EDTA (1 mM)	none	98
24	albumin (1 mg/ml)	none	105

unclear, but it is conceivable that the step 6 enzyme may be contaminated to varying degrees with proteins that would replace DTT. In support of this view is the finding that the step 2 or step 3′ enzyme, being much less susceptible to –SH reagents than the step 6 enzyme (Fig. 5), does not undergo this type of inactivation. In addition, reactivation can be achieved by EDTA or albumin as well as by DTT (Table III).

The kinetic properties of the step 6 enzyme that had been inactivated by a prior aging were studied in the absence and presence of DTT (Fig. 10). When DTT was absent, the enzyme failed to exhibit negative cooperativity and was inhibited by

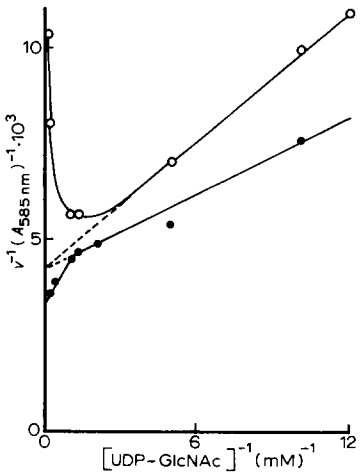

Fig. 10. Effect of aging on UDP-GlcNAc binding of 2′-epimerase. The step 6 enzyme left at 4 °C for 18 h in the presence of 0.1 mM UDP was assayed with varying concentrations of UDP-GlcNAc in the absence (●), or presence (○) of 1 mM DTT.

high substrate concentrations*. DTT, however, restored the negative cooperativity of the inactivated enzyme to a degree similar to that found for the fresh enzyme. Fig. 10 also shows that the inactivation is a consequence of the rise in K_m rather than fall in V.

The above inactivation was accompanied by a slight reduction in CMP-NANA inhibition which was also reversed readily by DTT. The inactivated enzyme thus resembles the 2′-epimerase samples described in previous studies[2,4–6] in that it is lower in cooperative interaction and in binding of substrate and allosteric ligands as compared with the fresh enzyme in the present study. Kornfeld *et al.*[2] reported that rat liver 2′-epimerase studied by them was inhibited by high concentrations of substrate.

DISCUSSION

The purification procedure described in this paper has yielded UDP-GlcNAc 2′-epimerase that differs strikingly from the previous preparations[2,4–7] in kinetic

* When the step 6 enzyme was inactivated by aging, the disappearance of negative cooperativity was followed by the appearance of substrate inhibition.

properties. The apparent K_m for UDP-GlcNAc of the present enzyme is 0.08 mM, a value that is much lower than those reported previously[2,4,6]. Furthermore, the present study has revealed a hitherto undetected negative cooperative interaction in the binding of UDP-GlcNAc to 2′-epimerase. The present enzyme is also much more sensitive than the previous preparations to inhibition by CMP-NANA and exhibited the Hill coefficient greater than that reported previously[6].

It is evident from these observations that the present enzyme is in a conformational state much closer to the native enzyme than are the previous preparations. One of the causes for this difference appears to be that in the present work, UDP was used instead of uridine as a stabilizing agent. Uridine exerts neither a stabilizing nor an inhibitory effect on 2′-epimerase, while UDP is a powerful competitive inhibitor as well as an effective stabilizer. Since UDP-GlcNAc is also as highly an effective stabilizer as UDP, UDP appears to exert its stabilizing effect through its capacity to combine with the substrate site reversibly.

Sommar and Ellis[7] reported previously that UDP inhibits 2′-epimerase. Their data, however, differ from ours in that the inhibition is of a linear non-competitive type thus consistent with an ordered uni-bi mechanism, where UDP is reversibly released prior to the irreversible formation of *N*-acetylmannosamine. If we assume that 2′-epimerase reaction takes place in the order proposed by them, our data then suggest that the release of UDP is almost irreversible, thereby excluding the possibility of simple product inhibition. These considerations lend support to our contention that UDP interacts with the enzyme at the substrate site owing to its structural similarity to the substrate. Glaser[16] and Salo and Fletcher[5] did not necessarily consider that the release of UDP was reversible.

The effect of DTT on 2′-epimerase is rather complex. At 4 °C, DTT (but not EDTA or albumin) could replace UDP in protecting the step 2 enzyme from irreversible inactivation (Fig. 1A). This effect of DTT, however, was no longer detectable with highly purified preparations such as the step 6 enzyme, on which UDP was still highly effective. Since DTT, but not UDP, failed to stabilize the step 2 enzyme at 37 °C (Fig. 2), the simplest interpretation appears to be that a specific conformational form might be required for 2′-epimerase to be protected by DTT. Such a conformational form would be difficult to maintain during the entire purification procedure or if the temperature rises from 4 °C to 37 °C.

For the purest step 6 enzyme, although the above effect of DTT was not apparent, the decrease in enzyme activity upon aging at 4 °C in the presence of UDP was reversed by DTT (or EDTA or albumin) (Table III). The loss in catalytic activity was due to an increase in the K_m for UDP-GlcNAc and was accompanied by some other alterations in UDP-GlcNAc binding such as the disappearance of substrate activation and appearance of substrate inhibition (Fig. 10).

The changes in UDP-GlcNAc binding seen here are probably due to reversible conformational changes in which the –SH groups on the enzyme would play a decisive role. The occurrence of such conformational changes would also explain, at least in part, marked discrepancies encountered in literature as to the binding properties of 2′-epimerase.

The anomalous kinetics exhibited by the fresh enzyme in UDP-GlcNAc binding has been shown to be negative cooperativity as defined by Conway and Koshland[14]. The possibility that the anomalous kinetics is due to two Michaelis–

Menten isoenzymes is excluded by the finding that the kinetics persists during purification but disappears reversibly upon aging at 4 °C or by UDP or CMP-NANA. These results suggest that 2′-epimerase possesses multisubstrate sites. The enzyme also possesses at least 6 CMP-NANA sites since the Hill coefficient of 5.7 was obtained for the CMP-NANA inhibition[17]. It is, therefore, reasonable to assume that 2′-epimerase is an oligomeric enzyme comprising at least 6 subunits.

The importance of 2′-epimerase as a regulatory enzyme resides in the fact that it is subject to feedback inhibition by CMP-NANA. Previous studies[2,6], however, reported that CMP-NANA could not inhibit the enzyme more than 60–70%. In addition, the concentration of CMP-NANA required for 50% inhibition (about 0.3 mM) was almost an order of magnitude greater than the intracellular concentration of this nucleotide.

Such a low sensitivity towards CMP-NANA is obviously a consequence of secondary modifications in the enzyme molecule, since the present study revealed that a concentration for half-maximal inhibition (0.025 mM) is in close agreement with the level of this nucleotide in rat liver (0.03–0.05 mM)[15]. Furthermore, the highly cooperative nature of CMP-NANA inhibition allows that a change in its concentration from 0.017 to 0.038 mM is sufficient to reduce 2′-epimerase activity from 90 to 10% of the maximal level (Fig. 8A). The rate of CMP-NANA synthesis *in vivo* would, therefore, be controlled by the inhibition of 2′-epimerase by CMP-NANA quite efficiently.

The occurrence of negative cooperativity in UDP-GlcNAc binding described above means that an increase in UDP-GlcNAc concentration above 0.5 mM augments 2′-epimerase activity. As the physiological level of UDP-GlcNAc is said to be approx. 0.3 μmole/g rat liver[2,18], this type of substrate activation may be of physiological significance. It is, therefore, appropriate that the two effective inhibitors of 2′-epimerase, UDP and CMP-NANA, bring about a release of the negative cooperativity.

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