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Substrate Stereochemical Requirements in the Reductive Inactivation of Uridine Diphosphate Galactose 4-Epimerase by Sugar and 5′-Uridine Monophosphate†

Jeanne Nelson Ketley‡ and Karl A. Schellenberg*

ABSTRACT: The reductive inactivation of uridine diphosphogalactose 4-epimerase (EC 5.1.3.2) by specific sugars in the presence of 5′UMP has been investigated as a model reaction for the first step in the epimerization of D-glucose to D-galactose. By following the stereospecific transfer of tritium label from sugar to enzyme, we have determined that the 4 position of D-galactose, but not D-glucose, is active in the reduction of enzyme. The 1 positions of both sugars are also active. The labeled compound dissociated from the enzyme was identified as B-[4-^{3}H]DPNH by its migration with DPNH on several chromatographic systems, as well as by incubation with specific dehydrogenases. A model is presented of the common stereochemistry of three isomeric centers around the position of hydrogen transfer in the sugar molecules that are active as model substrates.

Uridine diphosphate galactose 4-epimerase from *Escherichia coli* is a dimer containing a tightly bound DPN$^+$ (Wilson and Hogness, 1969) which catalyzes the intramolecular epimerization of UDP-D-galactose[1] to UDP-D-glucose (Glaser and Ward, 1970). Both yeast and *E. coli* epimerase have been shown to undergo a model reaction with free sugar and 5′UMP which results in the inactivation of enzyme and the net formation of tightly bound DPNH (Kalckar *et al.*, 1970; Bertland *et al.*, 1971). It has been speculated that this reaction represents the first step of the epimerization reaction (Davis and Glaser, 1971). It is reasonable to predict that in the model reaction the H from the 4 position of the sugar moiety is removed by DPN$^+$ in an oxidation–reduction reaction yielding DPNH and a 4-keto intermediate. Recent work utilizing 4-keto sugar nucleotide analogs (Nelsestuen and Kirkwood, 1971), as well as NaB^{3}H$_4$ trapping experiments in the presence of enzyme and UDP-galactose in which UDP-[4-^{3}H]hexoses were isolated (Maitra and Ankel, 1971), give strong support for the presence of a 4-keto intermediate in the normal reaction.

The relation of the model reaction to the complete one became ambiguous with the finding of Davis and Glaser (1971) that the reductive inactivation reaction shows an isotope effect upon incubation with [3-^{2}H]glucose, but not with [4-^{2}H]glucose. This implicated the 3 position in the epimerization. However, these workers observed no transfer of ^{3}H to enzyme upon incubation with [3-^{3}H]glucose.

Therefore, the object of these experiments was to test whether the proposed model reaction is reflective of the enzyme reaction, and if so, what are the stereochemical requirements of the enzyme for substrate. Also, we considered that, if the model reaction is indicative of the first step of the epimerization, then any other functional groups on the enzyme besides DPN$^+$ which might be involved in the reaction

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‡ Predoctoral Fellow of the Department of Physiological Chemistry.

[1] Abbreviations used are: uridine 5′-(α-D-galactopyranosyl pyrophosphate), UDP-galactose; UDP-D-glucose 4-epimerase (EC 5.1.3.2), UDP-galactose 4-epimerase.

would possibly be labeled. Either the involvement of another group on the protein or a conformational change of the protein could explain the intramolecular nature of the reaction.

During the course of these experiments Seyama and Kalckar (1972a,b) demonstrated the transfer of the C-1 hydrogen of D-galactose and D-arabinose to enzyme during the reduction of epimerase by 5′UMP and specific sugars. We have verified these results. Further, we have shown that B-DPNH is produced upon incubating epimerase with 5′UMP and [4-^{3}H]-galactose, but not by incubation with [4-^{3}H]glucose, [3-^{3}H]-glucose, or [2-^{3}H]glucose.

A model describing this asymmetric transfer from substrate to enzyme in terms of a common orientation of the sugar at the enzyme active site is described.

Materials and Methods

Enzymes and Microorganisms. UDP-galactose 4-epimerase was purified by a modification of the procedure of Wilson and Hogness (1964) in which the hydroxylapatite step was omitted, from *E. coli* strain D96 (*proC*$^-$, *ade*$^-$, *thi*$^-$) *gal* R^c, λdgqs, a culture containing a temperature-inducible λdg phage. The specific activity of the preparations used in these experiments ranged from 5×10^3 to 7×10^3 μmol per mg of protein per hr when measured at 27°.

UDP-galactose 4-epimerase activity was determined by measuring DPNH production after the addition of UDP-D-galactose to an incubation mixture containing excess DPN$^+$ and UDP-glucose dehydrogenase (Wilson and Hogness, 1964). Sodium dodecyl sulfate gel electrophoresis of these preparations revealed one major band comprising 95% or more of the protein present (Weber and Osborn, 1969). The bacteria were grown with maximal aeration at 30° in a New Brunswick Scientific fermentor containing 100 g of yeast extract, 150 g of bactotryptone, and 50 g of NaCl in a total volume of 10 l. When the bacterial density reached an optical density of 1.25–1.90, measured at a wavelength of 600 nm, the temperature was raised to 45°, then lowered immediately to 37° and the fermentation was allowed to continue for 3 hr. After this time the cells were centrifuged and the cell paste was stored at −15°.

UDP-glucose dehydrogenase was obtained from Sigma Co., and yeast alcohol dehydrogenase and glutamate dehydrogenase were obtained from Boehringer.

Radioactivity Determinations. Paper chromatograms were assayed for radioactivity with a Vanguard Autoscanner 880. Liquid scintillation counting was done in a Beckman LS-100 counter. A maximum sample volume of 0.5 ml was mixed with a scintillation solution containing 5 ml of absolute ethanol and 10 ml of 0.6% (w/v) of diphenyloxazole in toluene. The counting efficiency for ^{14}C in this fluid is approximately 50% and for ^{3}H, 13%.

Radiochemicals. D-[1-^{14}C]Glucose, D-[1-^{14}C]galactose, D-[4-^{3}H]glucose, D-[3-^{3}H]glucose, D-[2-^{3}H]glucose, and D-[4-^{3}H]galactose were obtained from Amersham/Searle. The radiochemical purity of all compounds was verified by paper chromatography of each sugar in system III followed by scanning and $AgNO_3$ spray visualization of the sugar spots. In all cases over 98% of the radioactivity was associated with the appropriate $AgNO_3$-positive spot. We further checked the position of ^{3}H in the sugars by periodate oxidation of the osazone derivatives (Aranoff, 1967). The phenylosazones were oxidized with periodic acid, yielding a 1,2-bis(phenylhydrazone) containing C-1, C-2, and C-3, formic acid from the C-4 and C-5, and formaldehyde from C-6. Lyophilization of the mixture from acid solution yielded formic acid and formaldehyde in the distillate, whereas lyophilization from neutral solution yielded only formaldehyde in the distillate. This method does not differentiate between C-4 and C-5, or C-1 and C-3 but, since it is unlikely that the C-5 hydrogen is involved in the epimerase reaction, and our major concern being the determination of possible D-[1-^{3}H]galactose contamination of the D-[4-^{3}H]galactose sample, we did not pursue the analysis further. The results of these experiments allowed us tentatively to conclude that the tritiated sugars as purchased are labeled as indicated. The C-1 tritium content was also determined by two separate methods, reaction of the sugars with glucose oxidase (Kusai *et al.*, 1960) and *N*-bromosuccinimide oxidation (Green, 1957) followed by determining the amount of ^3H_2O produced in these reactions. These experiments showed that 1% or less of the label of the 4-^{3}H-labeled sugars is at C-1, and indicated that all the sugars are essentially radiochemically pure.

Thin-Layer and Paper Chromatography. Thin-layer chromatography was performed using Polygram Cel 300 PEI plates (20 × 20 cm) obtained from Brinkmann Instruments, Inc., and developed with 0.2 M NH_4HCO_3. Paper chromatography was done on Whatman No. 1 or 3MM paper using the following solvents (v/v): system I, propanol–concentrated NH_3–H_2O (6:3:1); system II, absolute ethanol–1 M ammonium acetate (pH 7.5) (3:1); system III, ethyl acetate–pyridine–H_2O (10:4:3). The position of nucleotides on these chromatograms was determined by their ultraviolet absorption or fluorescence, the position of amino acids by the ninhydrin reaction, and the position of sugars by $AgNO_3$ spray (Anet and Reynolds, 1953).

Sephadex Column Chromatography. Sephadex G-25F columns were prepared with 20 mM K_2HPO_4, 1 mM mercaptoethanol, and 1 mM EDTA, and eluted either with this buffer or 50 mM NH_4HCO_3.

Chemicals. Yeast extract and Bactotryptone were obtained from Difco Laboratories. UDP-D-galactose, UDP-D-glucose, 5′UMP, and α-ketoglutaric acid were obtained from Sigma Co. DPN$^+$ and DPNH were from Boehringer.

Other Methods. Protein concentrations were determined by the Lowry method (Lowry *et al.*, 1951) and by absorbance at 280 nm. We found that a solution of pure epimerase with a protein concentration of 1 mg/ml as determined by the Lowry method had an OD_{280} of 1.04. A Beckman DU spectrophotometer modified with a Gilford photometer and a Sargent-Welch recorder were used in the spectral studies.

Results

^{3}H Labeling of UDP-galactose 4-Epimerase. UDP-galactose 4-epimerase (2–10 mg) was incubated with 20 mM D-galactose containing D-[4-^{3}H]galactose ($8–11 \times 10^7$ dpm in various experiments) and D-[1-^{14}C]galactose ($2.5–9.5 \times 10^6$ dpm) in the presence of 0.10 mM 5′UMP in 0.5 ml of 60 mM sodium glycine buffer (pH 8.5) containing 1 mM 2-mercaptoethanol and 1 mM EDTA, for 5 days at 4°. This is similar to the conditions used by Seyama and Kalckar (1972a,b) to achieve maximum reduction of bound DPN$^+$ as measured by fluorescence emission at 450 nm. After this time, the specific activity had fallen to 10–20% of that of a control mixture in which sugar was omitted. The incubation mixture was chromatographed on Sephadex G-25F. The protein peak had a higher ^{3}H:^{14}C ratio than the unbound sugar peak indicating that the protein was labeled by ^{3}H transferred from the sugar in addition to bound sugar (Figure 1).

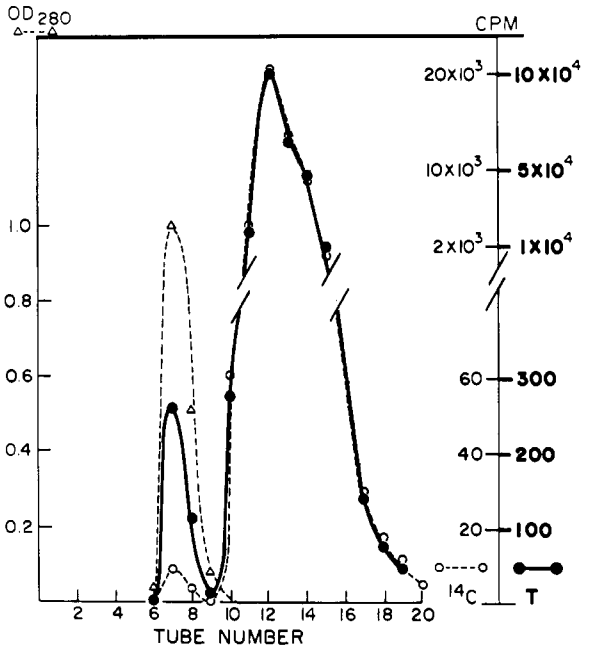

FIGURE 1: Sephadex G-25F chromatography after incubation with D-[4-^{3}H]galactose (8.71 × 10^7 dpm) and D-[1-^{14}C]galactose (4.91 × 10^6 dpm) and 5′UMP. The incubation mixture was placed on a Sephadex G-25F column (12 × 350 mm) and eluted with K_2HPO_4 buffer. Fractions of 1 ml were collected, the OD_{280} measured and then 2 drops of each fraction were added to scintillation fluid and counted for ^{3}H and ^{14}C. (●) ^{3}H, (○) ^{14}C, and (Δ) A_{280}.

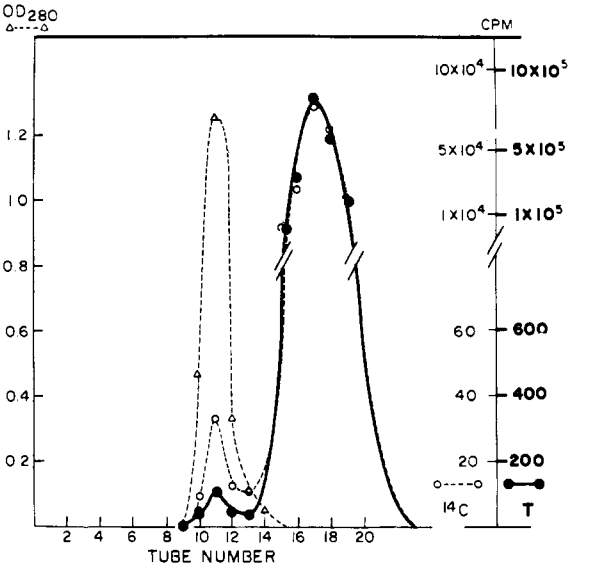

FIGURE 2: Sephadex G-25F chromatography after incubation with D-[4-^{3}H]glucose (1.44 × 10^8 dpm) and D-[1-^{14}C]glucose (3.05 × 10^6 dpm) and 5′UMP. Conditions were the same as described for Figure 1.

In contrast, when D-[4-^{3}H]glucose, D-[3-^{3}H]glucose, or D-[2-^{3}H]glucose at approximately the same tritium concentration replaced D-[4-^{3}H]galactose in an incubation mix, a ^{3}H:^{14}C ratio of 1 or less was observed indicating no ^{3}H labeling of the protein (Figure 2).

Table I is a summary of three experiments, described above, with minor variations. Also included (expt 4) are incubations using D-[1-^{3}H]galactose as the tritiated sugar. The ^{3}H:^{14}C ratio greater than one associated with protein indicates the protein has been labeled, a result which confirms those of Seyama and Kalckar (1972a). Another incubation utilizing D-[1-^{3}H]glucose shows that the protein can be labeled from the 1 position of D-glucose as well as from the 1 position of D-galactose. The incubations with D-[1-^{3}H]hexoses were not analyzed further.

The fractions containing protein were combined, an aliquot was taken for protein concentration and radioactivity measurements, and then they were stored under N_2 at 4° for 7 days. When rechromatographed on Sephadex G-25F, to separate any labeled sugar which had dissociated, the protein fraction from the D-[4-^{3}H]galactose incubation had only ^{3}H bound to it, whereas the protein fraction from the D-glucose incubations had no radioactivity above background associated with it (Figure 3).

Isolation of Cofactor from D-[4-^{3}H]Galactose-Labeled Protein. Preliminary experiments indicated that the ^{3}H label could be dissociated from the enzyme with heat, and absorbed by charcoal. Working on the assumption that the DPN^+ cofactor had been labeled, we added, to the combined protein fraction, 0.2 ml of a freshly made DPNH solution (10 mg/ml), and placed the mixture in boiling water for 2 min. This procedure sometimes resulted in a cloudy solution. The mixture was cooled and placed on a 20 × 310 mm column of Sephadex G-25F and eluted with 50 mM NH_4HCO_3. The absorbance of the fractions at 280 and 340 nm was determined. The tubes corresponding to the DPNH region were combined and lyophilized. The residue was dissolved in a small volume of water (0.5 ml, 2.36 × 10^4 cpm of ^{3}H) and used in the ex-

TABLE I: Relative Labeling of G-25F Protein Fractions by ^{3}H- and ^{14}C-Labeled Sugars.[a]

Expt	Sugar (+ ^{14}C-Labeled Sugar)	Incubn Time	pH	nmol Bound/nmol of Protein ^{3}H	^{14}C	^{3}H:^{14}C
1	[4-^{3}H]Galactose	6 days at	8.5	0.500	0.290	1.7
	[4-^{3}H]Glucose	4°		0.078	0.113	0.69
2	[4-^{3}H]Galactose	Same	8.5	0.580	0.424	1.4
	[4-^{3}H]Glucose			0.050	0.075	0.67
	[3-^{3}H]Glucose			0.272	0.296	0.92
	[2-^{3}H]Glucose			0.052	0.144	0.36
3	[4-^{3}H]Galactose	1 day at	7.5	0.114	0.058	2.0
	[4-^{3}H]Glucose	4°		0.023	0.081	0.28
	[3-^{3}H]Glucose			0.091	0.093	0.98
4	[4-^{3}H]Galactose	4 days at	8.5	0.292	0.057	5.1
	[1-^{3}H]Galactose	4°		0.348	0.083	4.2
	[1-^{3}H]Glucose			0.248	0.086	2.9

[a] The quantities are calculated from ratio of specific activity of protein fraction to that of added sugar. The amount of bound sugar is indicated by the ^{14}C ratio of protein to sugar. Any additional transfer of tritium is indicated by a ^{3}H:^{14}C ratio of greater than one. The reason for the ^{3}H:^{14}C ratio of less than one in some experiments is not known. It could be due to base-catalyzed enolization of free sugar during incubation, in which isomerization can take place leading to the loss of ^{3}H from the sugar (Pigman, 1957).

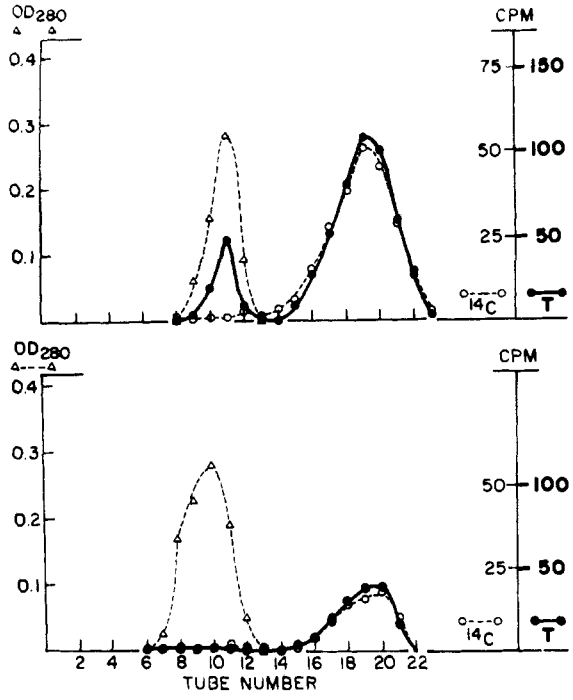

FIGURE 3: Rechromatography on Sephadex G-25F. (top) Of the protein fraction isolated from the experiment described in Figure 1. Chromatography conditions were the same as described for Figure 1. (bottom) Of the protein fraction isolated from the experiment described in Figure 2. Chromatography conditions were the same as described for Figure 1.

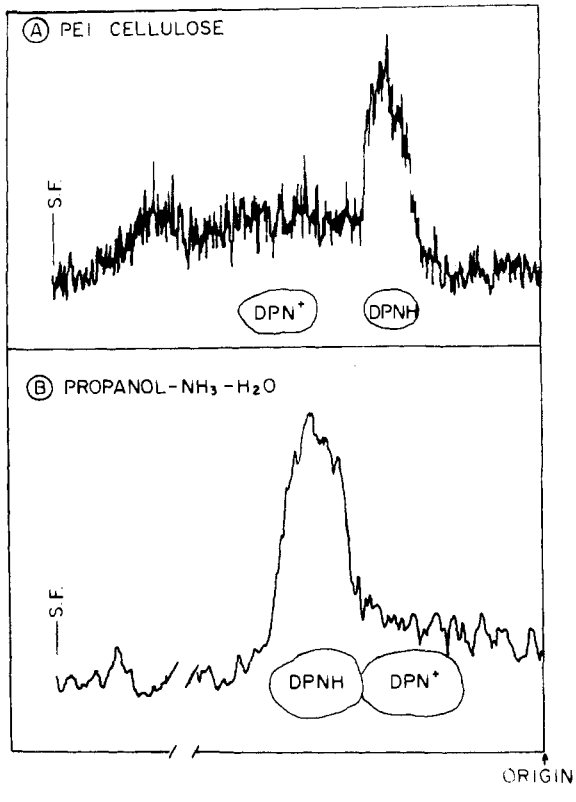

FIGURE 4: Chromatographic identification of isolated cofactor. (A) Radioactivity scan of thin layer chromatography on PEI-cellulose plates compared with known DPN^+ and DPNH spots. (B) Scan of paper chromatogram of unknown chromatographed in system I.

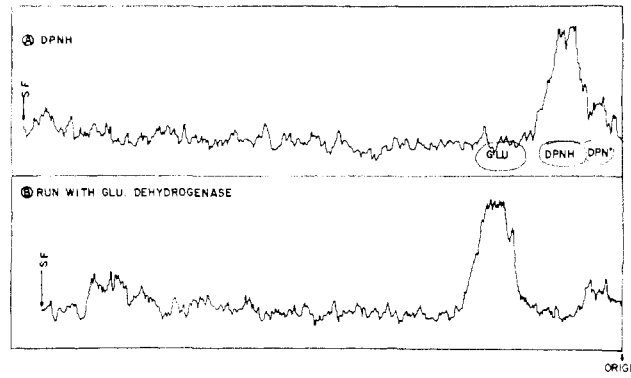

FIGURE 5: Chromatography of glutamate dehydrogenase reaction mixtures compared with standards. (A) Radioactivity scan of paper chromatogram developed with system II of the isolated cofactor. (B) Scan of paper chromatogram after the use of the unknown as a cofactor in a glutamate dehydrogenase reaction mixture.

periments described below to determine the position of the label.

Identification and Stereochemistry of Presumed Cofactor. The ^3H-labeled compound was found to migrate with DPNH on thin-layer chromatography and by paper chromatography in system I (Figure 4). In order to determine the absolute specificity of the labeling reaction, we incubated the presumed cofactor with A- and B-specific dehydrogenases. A portion of the labeled cofactor (0.1 ml) was reacted in an equilibrium mixture with 6 mg of yeast alcohol dehydrogenase, 7 mg of DPN^+, 0.05 ml of absolute ethanol, and 0.10 mM sodium pyrophosphate buffer (pH 8.3) in a total volume of 1 ml at 25° for 30 min, at which time it was frozen and lyophilized. There were 58 cpm in the reaction mixture distillate *vs.* 72 cpm in a control in which the dehydrogenase was omitted. Previous experiments with known A-DPN^3H have given complete equilibrium with ethanol. Approximately 2000 cpm would have been expected in the distillate if the material were A-DPN^3H. Reaction of a second portion (0.1 ml) with 0.005 ml of glutamate dehydrogenase (100 mg/ml in glycerol), 14 mM $(NH_4)_2SO_4$, 7.1 mM Tris buffer (pH 8.0), and 0.71 mM α-ketoglutarate in a total volume of 0.7 ml in a cuvet at 27° while following the optical density at 340 nm revealed the DPNH was depleted within 10 sec. The reaction was heated in a 110° oven for 1 min to inactivate the enzyme, then frozen, and lyophilized. The residue was dissolved in a small amount of water, spotted, and chromatographed in system II. A small amount of untreated isolated cofactor was also chromatographed in this system as a control. The results of this chromatography can be seen in Figure 5. There is an almost quantitative transfer of ^3H to glutamate. It is evident from these experiments that the incubation of UDP-galactose 4-epimerase with D-[4-^3H]galactose and 5'UMP results in the transfer of ^3H to the B side of the nicotinamide moiety of the enzyme-bound DPN^+.

Discussion

The bound DPN^+ of UDP-D-galactose 4-epimerase from *E. coli* has been labeled by the 4-H of the hexose moiety of the substrate analogs UDP-6-deoxyglucose (Nelsestuen and Kirkwood, 1971) and TDP-glucose (Gabriel *et al.*, 1972).

Our finding that the 4-H of D-galactose, as well as the 1-H, can reductively inactivate *E. coli* epimerase when incubated with 5'UMP is proof that the presumed model reaction does

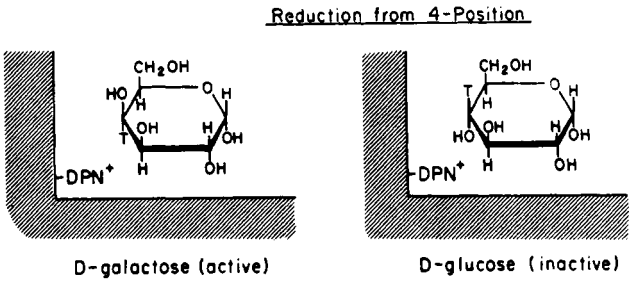

FIGURE 6: Haworth representations of D-galactose and D-glucose showing postulated orientation in relation to the tightly bound DPN^+.

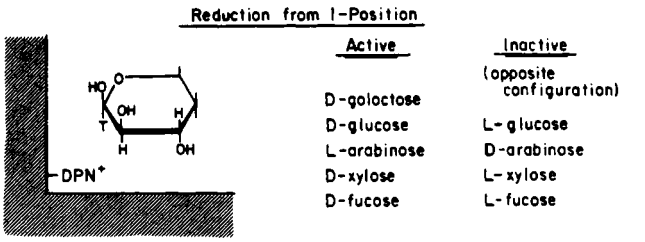

FIGURE 7: "Galactose-like" orientation of sugars that reduce epimerase from the 1 position.

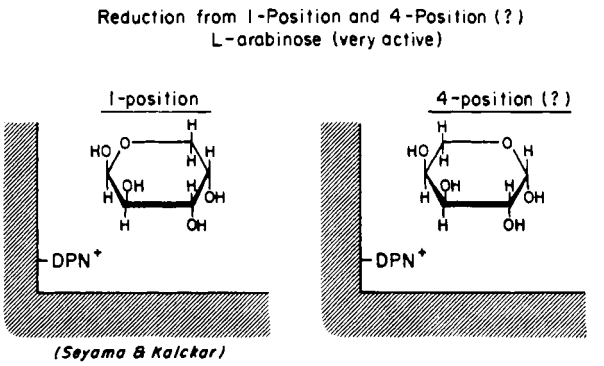

FIGURE 8: L-Arabinose in the two orientations which should allow transfer of H to DPN^+ according to the model presented. Transfer from the 1 position has been observed.

reflect a part of the overall catalytic epimerization of galactose to glucose. However, it is obvious that the sugar molecule can bind to the active site in either of two orientations, both of which yield reduced enzyme. The transfer of the 4-H of D-galactose is in agreement with the data of others presented earlier indicating that hydrogen abstraction from the 4 position of the sugar moiety of the substrate by the tightly bound DPN^+ is the first step in the epimerization reaction. Since the 1-H of the galactose moiety of UDP-galactose has been shown not to be transferred to the bound DPN^+ of epimerase (Seyama and Kalckar, 1972b), we can assume that the transfer from the one position is either an artifact of the model system or, as suggested by Seyama and Kalckar (1972a), plays a role in the *in vivo* regulation of epimerase.

The failure of glucose to transfer its 4-H, yet transfer its 1-H could be attributable to the resultant alterations in the secondary and tertiary structure of the enzyme due to the binding of the two-component substrate. After binding the conformation is such that the 4-H of glucose is inaccessible to the bound DPN^+. This hypothetical model is presented in Figure 6. Note the stereochemistry of C-2, -3, and -4 of D-galactose *vs.* D-glucose. In the case of reduction from the 1 position, if one makes the assumption that only the α configuration of the anomeric carbon can reduce the enzyme then by one rotation one can project a "galactose-like" relationship of sugar to enzyme on C-1, -2, and -3 of all the sugars that are known to probably utilize their reducing end to inactivate the enzyme (Figure 7). Also, their isomers which have been found not to reduce the enzyme, L-fucose, and D-arabinose (Kalckar *et al.*, 1970) and L-glucose and L-xylose (J. N. Ketley and K. A. Schellenberg, unpublished result), would have the opposite configuration to the model given.

Finally, we would predict that L-arabinose will not only reduce epimerase from the 1 position, as has been shown for yeast epimerase (Seyama and Kalckar, 1972a), but also from the 4 position (Figure 8). This substrate is the most active reducing sugar for yeast epimerase. We have found that it is also the most active sugar for the reduction of the *E. coli* enzyme. The enhanced rate of reduction by this sugar compared to the others could be a function of the facile binding to the enzyme due to its having the hydroxymethyl at C-6 substituted for by the smaller hydrogen atom.

The finding that *myo*-inosose-2, a ketoinositol with the same stereochemistry around C-2 and C-3 as the proposed 4-keto intermediate, can reactivate the reduced enzyme is in agreement with the model proposed (Ketley and Schellenberg, 1972b).

In conclusion, having observed no labeling of the protein besides the cofactor, we suggest that the asymmetric transfer of ^3H from D-[4-^{3}H]galactose, but not from D-[4-^{3}H]glucose to the enzyme DPN^+, implies a marked conformational change in order to effect the remainder of the epimerization catalyzed by the enzyme.

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On the Mechanism of Action of Streptococcal Proteinase. I. Active-Site Titration†

A. A. Kortt and T. Y. Liu*

ABSTRACT: The amount of active enzyme in proteinase solution has been determined by a number of methods including titration of the SH groups with 5,5′-dithiobis(2-nitrobenzoic acid) and stoichiometric inhibition of the enzyme with $HgCl_2$ or iodoacetic acid. The results of these experiments are consistent with the conclusion that the SH titer of the proteinase is a direct measure of the amount of active enzyme present. Proteinase preparations from different batches of crystalline zymogen exhibited different specific activities. The sole demonstrable difference was in their thiol content. Successful separation of active mercaptoproteinase and inactive nonmercaptoproteinase has been achieved by use of a Sepharose–mercurial column originally developed by Sluyterman and Wijdenes (Sluyterman, L. A. E., and Wijdenes, J. (1970), *Biochim. Biophys. Acta 200*, 593) for the purification of active papain. The active proteinase contained one titrable SH per mole of enzyme and exhibited a maximum specific activity, while the inactive proteinase contained less than 0.05 mol of SH/mol of enzyme and failed to exhibit any enzymic activity. These results are in agreement with the suggestion that the single SH group in the proteinase is essential for its enzymic activity. Sulfite ion was found to inhibit reduced proteinase at pH 5.0 at 25°. There was a binding of nearly 1 mol/mol of SO_3^- to the protein. The mechanism of this inhibition by sulfite ion is not yet known.

It has been shown (Elliott, 1945; Elliott and Dole, 1947) that group A streptococci elaborate an extracellular zymogen which can be transformed into an active proteinase by proteolysis followed by reduction. Both the zymogen (mol wt 44,000) and the enzyme (mol wt 32,000) contain only a single "potential half-cystine" residue per molecule (Liu *et al.*, 1963). The inactive enzyme formed by proteolysis of the zymogen requires activation by exposure to reducing agents. The zymogen isolated from the streptococcal culture is homogeneous by chromatography on an ion-exchange column, N-terminal residue analysis, amino acid analysis, and ultracentrifugal analysis (Liu *et al.*, 1963). Homogeneous samples of enzyme can be obtained in high yield upon treatment of the zymogen with trypsin, which removes about 100 amino acid residues (Liu and Elliott, 1965). The SH group of the potential half-cystine residue is liberated only when the enzyme is activated by reduction. This reductive step causes no detectable change in molecular weight.

The presence of one half-cystine residue in both the zymogen and the unreduced enzyme is well established (Liu and Elliott, 1965). Performic acid oxidation followed by acid hydrolysis of the zymogen or the unreduced enzyme after activation with thiols yields one residue of cysteic acid per mole. However, it has been known for some time that essentially homogeneous zymogen or unreduced enzyme after activation with thiols yields enzymes with different specific activities. When chromatographed on SE-Sephadex C-25 such activated proteinase showed heterogeneity (Liu and Elliott, 1965), but separation of active and inactive proteinase has not been achieved.

This article is concerned with experiments designed to demonstrate that the specific activity of the activated enzyme (proteinase) is directly proportional to its SH content. Separation of active mercaptoproteinase and inactive nonmercaptoproteinase was accomplished by use of the Sepharose–mercurial column of Sluyterman and Wijdenes (1970). A preparation was obtained which contains one titrable SH group per mole of proteinase. This preparation exhibited the maximum enzymic activity obtainable.

Experimental Section

Materials. Streptococcal zymogen was prepared by Dr. S. D. Elliott in the form of an 0.8-saturated $(NH_4)SO_4$ protein precipitate of the culture medium (Elliott, 1950). The papain preparation used in a control experiment was obtained from Worthington Biochemicals, crystalline suspension, lot PAP 9DA. The preparation showed 55% of the maximum activity obtainable when assayed with *N*-benzyloxycarbonylglycine *p*-nitrophenyl ester (Z-Gly-Nph).[1] Trypsin, twice crystallized, obtained from Worthington Biochemicals,

† From the Biology Department, Brookhaven National Laboratory, Upton, New York 11973. *Received August 28, 1972.* Research carried out at Brockhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

[1] The abbreviations used are: Z, benzyloxycarbonyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTE, dithioerythritol; Nph, *p*-nitrophenyl ester; Ph, phenyl ester.