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URIDINE DIPHOSPHOGLUCOSE DEHYDROGENASE FROM RAT LIVER: PURIFICATION AND EFFECT OF pH ON REGULATORY PROPERTIES

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SUMMARY

Uridine diphosphoglucose (UDPG) dehydrogenase (UDPglucose:NAD^+ oxidoreductase, EC 1.1.1.22) has been purified to apparent homogeneity from rat liver supernatant (105 000 × *g*). The enzyme was found to be NAD^+ specific and had maximal velocity at pH 9.4. It was also fairly active at pH 8.6 and under these conditions, it was allosterically inhibited by UDPxylose. However, at pH 9.4 the inhibition by this compound was competitive in nature. In the absence of the coenzyme, the enzyme could bind the substrate rather firmly. The binding of the substrate at pH 9.4 was accompanied by alterations in the protein resulting in partial dissociation of subunits as could be seen from the elution profiles on Sephadex G-100. The addition of the coenzyme prevented these changes. The enzyme showed an approximate molecular weight of 300 000 and seemed to be tetrameric after sodium dodecyl sulphate treatment. The kinetics of the enzyme in the native form and the dissociated state showed variations in affinity for the substrate with the respective K_m values of $7.56 \cdot 10^{-5}$ M and $1 \cdot 10^{-3}$ M. This was in keeping with the amount of substrate ([U-^{14}C]UDPG) retained on the kinetically inactive enzyme in undissociated and dissociated forms. The results show that the pH-dependent loss of regulatory property was related to the substrate-induced partial disaggregation of the protein.

INTRODUCTION

The enzyme uridine diphosphoglucose (UDPG) dehydrogenase (UDPglucose: NAD^+ oxidoreductase, EC 1.1.1.22) is not only involved in the biosynthesis of glucuronic acid[1] but also mediates in the initial reaction of the glucuronic acid cycle[2] and the biosynthesis of ascorbic acid[3]. The peculiar kinetics of this NAD^+ linked conversion of UDPG to uridine diphosphoglucuronic acid (UDPglucuronic acid) has attracted several investigations on the mechanism of its action[4–12]. It has been shown that for 2 moles of NAD^+ reduced, approx. 1 mole of UDPglucuronic acid was obtained[4–6,11]. It has been presumed that any intermediate involved in this four electron transfer reaction must remain tightly bound to the enzyme[4].

Despite its ubiquitous occurrence[1], UDPG dehydrogenase has been purified from relatively few sources[4,5,13–16]. The enzyme from calf liver which has been investigated in fair detail has been prepared in a high state of purity[9,16]. An attempt has been made in this paper to purify and characterise UDPG dehydrogenase from rat liver in which the presence of glucuronic acid as well as ascorbic acid synthesising mechanisms are recognised[2,3]. It has been shown that the enzyme can exhibit two conformational states differing in kinetic and other properties depending on pH.

MATERIALS AND METHODS

Materials

All biochemicals including UDPG, UDPglucuronic acid, UDPxylose, NAD^+ and $NADP^+$ and DEAE-cellulose (0.9 mequiv) were obtained from Sigma Chemicals Co. [U-^{14}C]UDPG was purchased from Calbiochem and Sephadex G-100 resin from Pharmacia. All other chemicals of highest purity were procured from standard sources.

Preparation of tissue extract

Male rats (Wistar) weighing 200–250 g fed on laboratory stock diet were sacrificed by cervical dislocation. The liver was excised, chilled and homogenised in 0.25 M sucrose to make a 10% suspension. The homogenate prepared from 30–40 g of rat liver was centrifuged at 45 000 rev./min (105 000 × *g*) for 1 h in a Beckman Model L-2-65B preparative ultracentrifuge, and was used as the enzyme source.

Assay of UDPG dehydrogenase

The enzyme activity was estimated by the method of Strominger *et al.*[4]. The reaction mixture consisted of 1.0 ml of 0.1 M glycine–NaOH buffer (pH 9.4) containing 1.5 μmoles of UDPG, 1.0 μmole of NAD^+ and 0.1 ml of enzyme extract. The incubation was carried out in a cuvette at room temperature and the increase in absorbance at 340 nm was measured in a Shimadzu Model QV-50 spectrophotometer. The unit of enzyme activity has been expressed as the amount of enzyme required to give an increase in absorbance of 0.001 per min under the conditions employed.

Protein estimations

Protein estimations were carried out spectrophotometrically[17]. In some determinations the method of Lowry *et al.*[18] was employed, with bovine serum albumin as standard.

Other analytical methods

The absorption spectral analyses of the purified enzyme with or without the substrate were carried out on a Perkin–Elmer DB Model 124 spectrophotometer coupled with Hitachi Model 165 recorder.

The radioactivity was estimated on a Beckman Model LS-100 Liquid Scintillation Spectrometer. The composition of the scintillation liquid was as stated by Bray[19].

EXPERIMENTAL AND RESULTS

Purification of rat liver UDPG dehydrogenase

All operations were carried out at 0–4 °C and centrifugations were performed in a Sorvall RC-2B refrigerated centrifuge using SS-34 rotor at 15000 rev./min for 30 min. Table I summarises the activities and recoveries of the enzyme at various steps employed for purification.

TABLE I

PURIFICATION OF RAT LIVER UDPG DEHYDROGENASE

	Volume (ml)	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg)*	*Recovery (%)*
Rat liver supernatant (105 000 × *g*)	260.00	3689	36 400	9.86	100
Ammonium sulphate (25–50% saturation)	50.00	633	20 000	31.60	55
Dialysis	57.00	633	25 935	40.80	71
Heat treatment	60.00	288	21 600	75 00	59
DEAE-cellulose	31.50	55	20 900	380.00	57
Sephadex G-100	15.00	7.9	16 400	2075.00	45

Ammonium sulphate fractionation. The supernatant obtained from rat liver homogenates, prepared as stated above, was brought to 25% saturation by adding solid $(NH_4)_2SO_4$ and was allowed to stand for 30 min. The precipitate obtained after centrifugation was discarded and the supernatant was brought to 50% saturation by further addition of the salt. The precipitate obtained after standing for 1 h was centrifuged and dissolved in 0.1 M sodium phosphate buffer (pH 6.2).

Dialysis and heat treatment. The enzyme thus prepared was dialysed against the same buffer for 3 h by making changes at 1 h intervals. The apparent increase in the recovery of the enzyme after dialysis indicated that the salt inhibited the activity.

The samples from different batches were pooled and the pH was adjusted to 5.5 using dilute acetic acid. The enzyme solution was then heated in a water bath at 54 °C for 2 min and quickly cooled in ice. The precipitate formed during this treatment was discarded and the solution rich in enzyme activity was lyophilised.

DEAE-cellulose chromatography. The lyophilised preparation (288 mg) was dissolved in the buffer and batches containing 50 mg of protein were loaded on the DEAE-cellulose column. Elution was carried out with sodium-phosphate buffer pH 6.2 and with a linear gradient of NaCl (0.05–1.0 M) as shown in Fig. 1. The enzymatically active samples were pooled and lyophilised.

Sephadex G-100 gel filtration. The lyophilised powder (50 mg) was dissolved in 5 ml of phosphate buffer pH 6.2 and then chromatographed on Sephadex G-100 column (2.1 cm × 60 cm). The elution was carried out for 3 h and 5-ml fractions were collected in an automatic fraction collector (LKB Ultro-Rak). The enzyme was eluted in a single peak which on rechromatography presented the profile shown in Fig. 2.

The purity of the preparation thus obtained was tested on polyacrylamide disc gel electrophoresis as shown in Fig. 2 by staining with amido black[20]. The UDPG

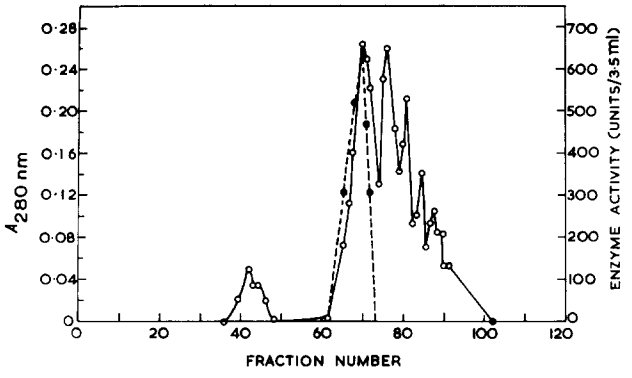

Fig. 1. Chromatography on DEAE-cellulose. The preparation of rat liver UDPG dehydrogenase obtained after heat treatment (50.0 mg protein) was applied to the column (1 cm × 50 cm). The elution was carried out using 0.1 M sodium phosphate buffer (pH 6.2) and a linear gradient of NaCl between 0.05–1.0 M. Fractions of 3.5 ml were collected. The enzyme activity was recovered in the tube Nos shown by the dotted line.

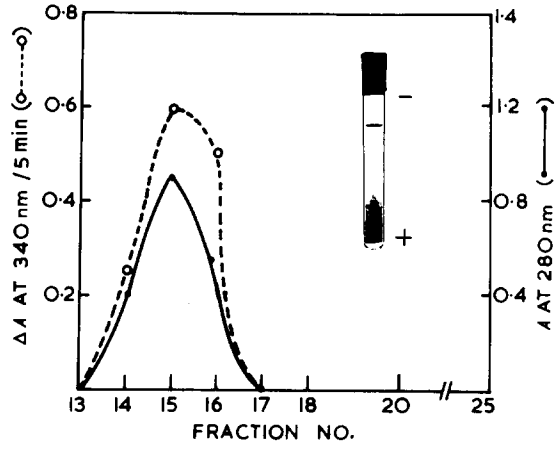

Fig. 2. Gel filtration of rat liver UDPG dehydrogenase on Sephadex G-100. The enzyme protein (8.0 mg) dissolved in 2.0 ml of 0.1 M sodium phosphate buffer pH 6.2 was loaded on to a column (2.1 cm × 60 cm) previously equilibrated with the buffer. Elution was carried out with the same buffer, collecting 5-ml fractions. The inset shows the polyacrylamide-gel electrophoretic pattern using 5 mA current per tube for 40 min at room temperature. The protein (100 μg) was applied to a 7.5% acrylamide gel.

dehydrogenase activity was also located in the same band by staining with phenazine methosulphate and nitroblue tetrazolium[14].

Properties of the enzyme

Rat liver UDPG dehydrogenase seemed to have two pH optima as shown in Fig. 3. Though the velocity of the reaction was maximal at pH 9.4, appreciable activity was also apparent at pH 8.6. For lower ranges between pH 6–8, sodium phosphate buffer (0.1 M) was used while for pH values 7.2–9.0 Tris–HCl buffer and for 8.6–10.0 glycine–NaOH buffer of the same molarity was employed.

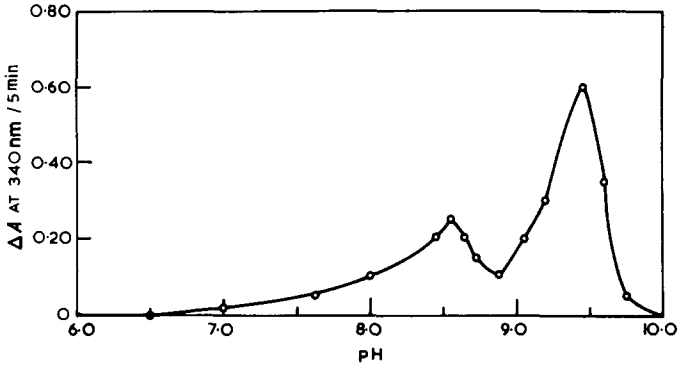

Fig. 3. Dependence of rat liver UDGP dehydrogenase on pH.

The enzyme activity was not affected by EDTA in the concentrations 10^{-5}–10^{-4} M indicating that there was no metal ion requirement.

$NADP^+$ could not replace NAD^+ for the hydrogen transfer involved in the reaction.

The molecular weight of the purified UDPG dehydrogenase was determined to be 300 000 using Sephadex G-100[21]. The standard proteins used in these determination were fibrinogen (340 000), bovine serum albumin (69 000), yeast hexokinase (96 000) and cytochrome *c* (12 800) as shown in Fig. 4.

To obtain an insight into the subunit structure of the protein, UDPG dehydrogenase was treated with 1% sodium dodecyl sulphate in 0.1 M sodium phosphate buffer, pH 7.2; the mixture was then incubated at 37 °C for 3 h[22]. This was then

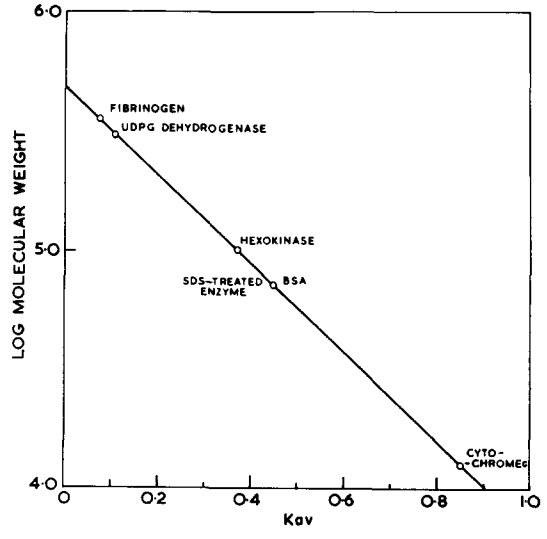

Fig. 4. Molecular weight determination of rat liver UDPG dehydrogenase using Sephadex G-100. The sample loaded and the column size are as stated in Fig. 2. The elution volume (V_e) for each protein as stated in the text was measured, the bed volume (V_t) and void volume (V_0) being 207 and 59 ml, respectively. Flow rate was maintained at 0.6 to 0.7 ml per minute. K_{av} was calculated as $(V_e - V_0)/(V_t - V_0)$. SDS = sodium dodecyl sulphate; BSA = bovine serum albumin.

passed through Sephadex G-100 column previously equilibrated with 1% of the detergent. The single peak that showed up indicated an apparent molecular weight of 70 000 as indicated in Fig. 4. It therefore seemed that the enzyme was tetrameric and possessed equal sized subunits.

Enzyme kinetics

The time course of activity at pH 9.4 showed that the enzyme reaction was almost complete within 3 min with the entire UDPG in the assay system being stoichiometrically converted to UDPglucuronic acid as determined by the orcinol method[23].

The apparent K_m (Michaelis constant) of the rat liver enzyme at pH 9.4 calculated[24] from the rate of reaction with different concentrations of UDPG, keeping NAD^+ level constant at 1.0 μmole/ml, was found to be $7.56 \cdot 10^{-5}$ M. The K_m (NAD^+) was similarly determined to be $3.39 \cdot 10^{-4}$ while keeping UDPG level at 1.5 μmoles/ml.

Allosteric nature of the enzyme

The earlier studies with calf liver UDPG dehydrogenase have indicated that UDPxylose exerts allosteric inhibition on the enzyme[13]. Therefore, the effect of this compound on rat liver UDPG dehydrogenase was ascertained. The varying concentrations of UDPxylose were added to the assay mixture as shown in Fig. 5. It was observed that co-operative interactions were apparent only at pH 8.6. The Hill plot made of UDPxylose inhibition at pH 8.6 is presented in the inset of Fig. 5. The Hill coefficient calculated as the slope of the plot[25] was found to be 2.2 suggesting the number of interacting sites[28]. However, at pH 9.4 the enzyme was inhibited competetively in a linear fashion with increasing concentrations of UDPxylose with a K_i of $9 \cdot 10^{-5}$ M.

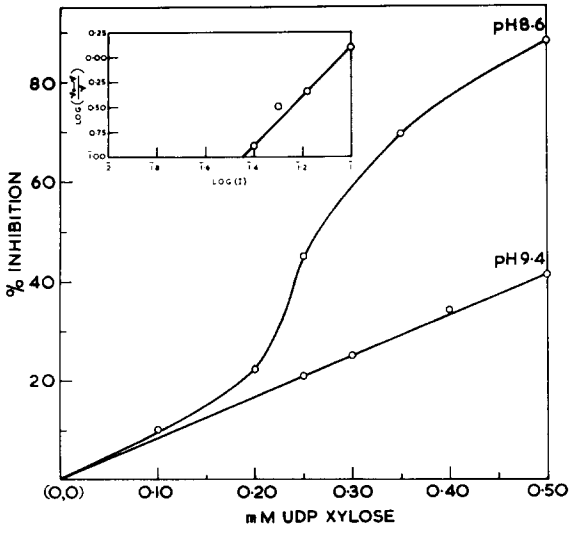

Fig. 5. The inhibition of rat liver UDPG dehydrogenase by UDPxylose. The responses have been determined at pH 8.6 and 9.4, respectively, as indicated. The inset shows a Hill plot with reference to varying concentrations of the inhibitor at pH 8.6.

Enzyme–substrate interaction

Earlier studies carried out with calf liver UDPG dehydrogenase presumed that the substrate becomes firmly bound to the enzyme and the product UDPglucuronic acid is released only after the reaction is complete[4–8,12]. An attempt was therefore made to assess the enzyme–substrate interaction with the rat liver preparation. The experiments were performed at pH 6.2, 8.6 and 9.4, respectively.

The enzyme preparation (3 mg) was dissolved in 0.1 M phosphate buffer, pH 6.2 or 8.6, or 0.1 M glycine–NaOH buffer, pH 9.4. The mixture was incubated at room temperature for 10 min with addition of UDPG (15 μmoles). It should be noted that NAD^+ was not added in these experiments, so that the enzyme reaction would not proceed and the protein would be functionally static. The mixture (1.5 ml) was then passed through Sephadex G-100 column (60 cm × 2.1 cm) with elutions carried out with the same buffer in which the enzyme preparation was initially dissolved. The fractions (5 ml each) were collected for a 3-h period using an automatic fraction collector (LKB ULTRO Rac). The absorbance of the collected samples was measured at 260 and 280 nm. In preliminary experiments, absorbance spectra of components in the mixture were recorded individually in the range 200–370 nm. It was observed that UDPG gave a maximum at 260 nm while the enzyme preparation gave an absorbance maximum at 280 nm.

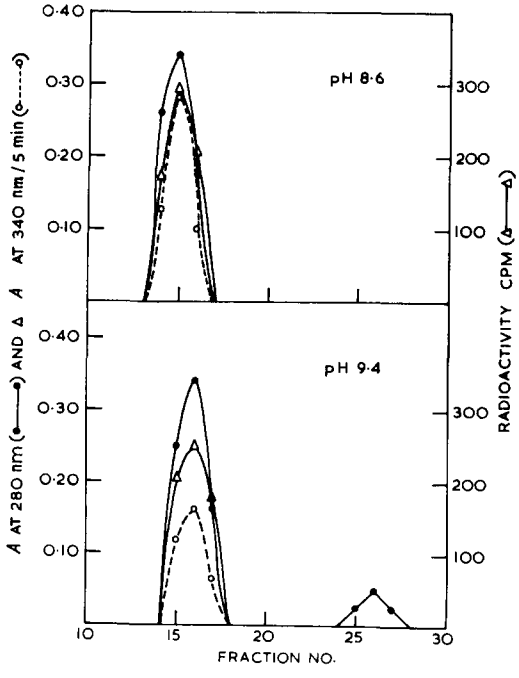

Fig. 6. Elution profiles of UDPG dehydrogenase on Sephadex G-100 in the presence of substrate at pH 8.6 and 9.4. The details are stated in text. The protein was estimated spectrophotometrically by measuring the absorbance at 280 nm and the enzyme activity as changes in absorbance at 340 nm. In radioactivity experiments [U-^{14}C]UDPG (15 μmoles) containing $2.8 \cdot 10^5$ cpm was added to 3 mg of the enzyme prior to gel filtration. The absorbance at 260 nm of the substrate in different tubes was observed to be concurrent with the radioactivity observed in these samples; hence the former has not been shown in this figure.

At pH 6.2 the enzyme and UDPG were eluted separately from the Sephadex G-100 column. The protein component separating in tube Nos 14–16 was enzymatically active when incubated with UDPG and NAD^+ in glycine–NaOH buffer, pH 9.4. The substrate appeared essentially in tube Nos 40–45 (not shown). The data, therefore, did not indicate any binding of the substrate when the mixture was chromatographed at pH 6.2. However at pH 8.6 a part of UDPG in the mixture eluted out with the protein component as shown in Fig. 6, thus showing formation of enzyme-substrate complex. This was confirmed by using [U-^{14}C]UDPG (1.0 μCi) in the experimental conditions as described above. The radioactivity was scanned in all the fractions obtained from the Sephadex G-100 column and was observed to be bound to the enzymatically active peak.

The profile obtained at pH 9.4 was different since at least two protein peaks were eluted in different fractions (Fig. 6) and only one of these was enzymatically active. The approximate molecular weights of these separating protein components were observed to be 230 000 and 70 000, respectively, pointing to the probability that one of the subunits of this tetrameric protein was becoming detached. The substrate was eluted with the active component indicating that some of the UDPG was firmly bound to the protein. When the enzyme alone was passed through Sephadex G-100 column at this pH, there was no apparent dissociation of the protein, suggesting that these alterations were related to the binding of the substrate. The addition of NAD^+ to the mixture also prevented this disaggregation.

Experiments were also carried out to see if NAD^+ alone could bind the enzyme using the same method as stated above, in which NAD^+ (but not UDPG) was added. We could not, however, observe any binding of the coenzyme in the experimental conditions employed.

Differences in the native enzyme and the dissociated protein

The K_m values of rat liver UDPG dehydrogenase and the enzymatically active component of the protein separating after dissociation (Fig. 6) showed significant differences. The K_m value of the dissociated protein for the substrate (UDPG) was found to be $1.0 \cdot 10^{-3}$ M which was approx. 13 times higher than that of the native enzyme as stated above, suggesting reduced affinity of the enzyme for the substrate.

The amount of radioactive substrate retained on the enzyme was assessed to be 3.7 and 2.6 moles per mole of the enzyme in undissociated and dissociated states, respectively, during the procedures described above. The substrate binding therefore appeared to be one equivalent per subunit.

DISCUSSION

The extent of purification of rat liver UDPG-dehydrogenase accomplished in the present studies points to apparent homogeneity of the preparation. The activity could not be detected with whole homogenates but became apparent only in the supernatant fraction as reported for calf liver[4–7] and rabbit skin[15]. The properties of the present preparation were generally similar to those of UDPG dehydrogenase reported from other sources, though it exhibited a peculiar pH effect. Thus, like UDPG dehydrogenase from *Aerobacter aerogenes*[14], the rat liver enzyme operated with maxi-

mum velocity at pH 9.4 at which point the inhibition exerted by UDPxylose was also of a competitive nature. However, at pH 8.6 it displayed the characteristics similar to those of the calf liver enzyme[4] when the interaction of the inhibitor with the enzyme was cooperative[13]. UDPG dehydrogenase from various sources such as pea seedling, bovine liver, chick cartilage and from *Cryptococcus laurentii*[26] has been reported to have allosteric properties; the only exception being that of *Aerobacter aerogenes*[14]. It was suggested that UDPxylose which potently and specifically acted on the enzyme served as a feed-back inhibitor to regulate the synthesis of UDPglucuronic acid[26]. The metabolic intermediates of galactose were also involved in controlling the enzyme activity in calf liver[27].

Several models that have been proposed to rationalise regulatory behaviour of enzymes envisage a conformational change of some kind in the protein[28]. It has now been well recognised that separate and specific sites exist for substrate and modifier[29]. It was observed that the abolition of CTP modulating action on aspartate transcarbamylase produced a striking decrease in the rate of sedimentation of the enzyme[30]. This was attributed to dissociation of the protein producing a catalytic subunit exhibiting normal Michaelis kinetics but insensitive to CTP and a regulatory unit having no catalytic activity but binding CTP. Recombination of these subunits restored the sigmoidal kinetics as well as CTP sensitivity[30]. The present experiments show a similar phenomenon in respect of UDPG dehydrogenase. It is apparent that the loss of cooperative interaction with UDPxylose at pH 9.4 could be correlated with dissociation of the enzyme protein (Fig. 6). Under the conditions, this compound would essentially act as the structural analogue of the substrate competing for the same site. Attempts to recombine the subunits to restore the native characteristics of the enzyme were not however successful with the techniques employed.

The allosteric enzymes should necessarily have two states which differed in affinities for the substrate[31]. The differences in K_m values of UDPG dehydrogenase in native and dissociated forms were thus in keeping with the presence of a polymerising protein system in which polymer and dissociated species differed in binding properties[32–35]. The present enzyme seemed to be tetrameric and could undergo changes only when the substrate became bound to the protein in the catalytically inactive state in the absence of the coenzyme. The binding ligand could distort the conformation of the enzyme thus affecting the stability and shape of the protein. The subunit interactions involved in these alterations have since been analysed to explain cooperativity and kinetic effects[36–38].

The dependence of UDPG dehydrogenase on pH for reactivity of the substrate has also been recognised[7–10]. The kinetic studies with 5-fluorouracil and 6-azauracil analogues of UDPG have suggested that the ionic state of the pyrimidine moiety had a determining effect on the binding of UDPG to the enzyme and that the undissociated form of the substrate was the effective species for interaction with the enzyme[7]. Though the involvement of the uracil moiety has been suggested in binding of UDPG to the enzyme, UDPG dehydrogenase was reported to be relatively unspecific for the nucleoside part of the substrate but was highly specific for the glucosyl portion. The glucose moiety in UDPG, dihydroxy-UDPG and 5-hydroxy-UDPG was oxidised to glucuronate at the same rate as NAD^+ was reduced[10]. The mechanism of action of the enzyme with reference to stepwise changes in the glucosyl moiety with the formation of UDP-gluco-hexodialdose have recently been clarified[12]. These studies also show

that the substrate existed in a bound state with the enzyme until the reaction was complete.

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