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Studies on the unusual behaviour of bovine liver UDP-glucose dehydrogenase in assays at acid and neutral pH and on the presence of tightly bound nucleotide material in purified preparations of this enzyme

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Assays of UDP-glucose dehydrogenase at pH 6.0 show long (10–15 min) lag periods before the steady-state rate is established, but at pH 9.0 no lag is observed. At intermediate pH values the lag is progressively shorter as the pH becomes more alkaline. The behaviour of the enzyme in assays at neutral and acid pH depends on the pH and concentration of the enzyme used to initiate the assay. The steady-state rate at pH 6.0 is strongly concentration-dependent. It is suggested that these phenomena arise because of the slow dissociation of an inactive enzyme species to an active one. Purified preparations of the enzyme release approx. 1 mol of a UDP-sugar/mol of enzyme subunit on denaturation. The identity of the UDP-sugar is unknown.

INTRODUCTION

Work from this laboratory in recent years has been concerned with the kinetics and mechanism of liver and yeast aldehyde dehydrogenases (Allanson & Dickinson, 1984; Dickinson, 1985, 1986; Dickinson & Haywood, 1986, 1987). It was decided to make a study of UDP-glucose dehydrogenase because this enzyme has an aldehyde dehydrogenase activity towards UDP-α-D-glucohexodialdose (Ridley *et al.*, 1975; Eccleston *et al.*, 1979) and the mechanism proposed for this oxidation is the same as that suggested for aldehyde dehydrogenase (see, e.g., Feingold & Franzen, 1981; Dickinson, 1986). Preliminary work showed that the purified enzyme showed unusual concentration-dependent assay characteristics that were reminiscent of those seen for sheep liver mitochondrial aldehyde dehydrogenase (Hart & Dickinson, 1978; Allanson & Dickinson, 1984). Further investigations indicated that the purified enzyme contained tightly bound non-protein material in significant concentrations. As neither of these properties appeared to have been reported previously, the work was pursued further and the results are recorded in the present paper.

EXPERIMENTAL

Materials

NAD^+ (grade II) was from Boehringer Corp., London W.5, U.K. UDP-glucose and other UDP-sugars were obtained from Sigma Chemical Co., Poole, Dorset, U.K. 5-{[(Iodoacetamido)ethyl]amino}naphthalene-1-sulphonic acid was also obtained from Sigma Chemical Co. Other chemicals were of analytical grade, where possible, and were obtained from Fisons Chemicals, Loughborough, Leics., U.K.

Methods

Enzyme. UDP-glucose dehydrogenase was prepared from freshly killed bovine liver by the method of Zalitis & Feingold (1969). In some instances the final product of the procedure was subjected to hydrophobic chromatography on phenyl-Sepharose at pH 7.0 with a reverse $(NH_4)_2SO_4$ gradient. This procedure produced a modest (approx. 10%) increase in specific activity. The enzyme preparations displayed only one band of M_r 53000 on SDS/polyacrylamide-gel electrophoresis and appeared to be completely homogeneous on passage through an analytical gel-filtration column (TSK G3000-SW; 8 mm × 300 mm) in 50 mM-sodium phosphate buffer, pH 7.0. The enzyme is normally stored in 50 mM-sodium phosphate buffer, pH 7.0, containing 0.3 mM-EDTA and 1 mM-dithiothreitol.

Activity assays. Routine assays of the enzyme were done under the conditions of Zalitis & Feingold (1969), but the reactions were generally monitored fluorimetrically with a filter fluorimeter of the type described by Dalziel (1962). Being a more sensitive method, the fluorimeter assay avoids the problems of product inhibition as NADH and UDP-glucuronate accumulate. For assays performed under other conditions the details are recorded in the text.

Studies on enzyme-bound nucleotide

Before the release of the nucleotide by boiling or by $HClO_4$ precipitation the enzyme was dialysed for 24 h versus a large excess of glass-distilled water. Any slight precipitate forming was removed by centrifugation.

Ion-exchange chromatography. This was carried out on a TSK-DEAE-5PW analytical column connected to an LKB f.p.l.c. system with monitoring at 254 nm. Elution protocols were performed under programme control at a flow rate of 0.8 ml/min at room temperature. For analytical work sodium phosphate buffers at pH 7.0 were used and the column was equilibrated with 50 mM buffer. Following the addition of 20–50 μl of sample the column was washed for 10 min with 50 mM buffer and then a linear gradient, taking 20 min to complete, was run

with 50–200 mM buffer. For larger-scale purification of material 200 μl samples were added to the column equilibrated with 30 mM-NH_4HCO_3. A 20 min 30–200 mM-NH_4HCO_3 linear gradient was applied immediately. On completion of the run the column was re-equilibrated at the lower NH_4HCO_3 concentration and the procedure was repeated. Appropriate fractions from successive runs were bulked together for freeze-drying.

Reversed-phase h.p.l.c. This was carried out on a 5 μm-particle-size Techsil RP 18 column of dimensions 250 mm × 4 mm. The procedure was based on that of Rottlán *et al.* (1986). Column development was with 0.5 M-potassium phosphate buffer, pH 6.0, at 40 °C and a flow rate of 1 ml/min. The effluent was monitored at 262 nm.

High-voltage paper electrophoresis. This was carried out on a Shandon and Southern model L24 apparatus. Samples (20 μl) of nucleotide solutions of $A_{260}^{1\,cm}$ in the range 6–13 were applied. Electrophoresis was for 1 h at 3 kV in 50 mM-potassium phosphate/citrate buffer, pH 3.5.

Phosphate analysis. Samples containing 10–40 nmol of phosphate were ashed with $Mg(NO_3)_2$ and assayed as described by Ames & Dubin (1960).

RESULTS AND DISCUSSION

Behaviour of UDP-glucose dehydrogenase in assays at acid and neutral pH

Fig. 1(*a*) shows the results of fluorimetric assay of UDP-glucose dehydrogenase at pH 6.0 and pH 9.0 with an enzyme concentration of 2.5 μg/ml. The enzyme used to initiate each reaction was maintained at a concentration of 1 mg/ml for 5 min at the same temperature and in the same buffer as used in the assay. At pH 9.0 the reaction proceeds immediately and rapidly, but there is a very small lag (approx. 10 s) at the start. At pH 6.0, on the other hand, there is no detectable reaction for several minutes, after which there is a very slow approach to the steady-state rate. The behaviour at pH 6.0 is unaffected by change to potassium phosphate or Tris acetate buffers or by inclusion of 1 mM-EDTA and/or 1 mM-dithiothreitol. It seems clear from the fact that the initial rate is apparently zero at pH 6.0 that the subsequent increase in rate cannot be due to product activation. This is confirmed by the fact that including the products, either separately or together and at appropriate (1–2 μM) concentrations, does not alter the profile when enzyme is added to start the reaction.

The behaviour of the enzyme in assays is affected by the pre-incubation conditions. As shown in Fig. 1(*b*), if the pH 6.0 assay is initiated by enzyme kept at pH 9.0 there is now almost no lag, the low steady-state rate being rapidly established. On the other hand, if the pH 9.0 assay is initiated with enzyme pre-incubated at pH 6.0, the resulting progress curve is very similar to that of an assay at pH 9.0 initiated with enzyme kept at pH 9.0. There may, however, now be a short (0.1 min) lag phase. If enzyme from the pH 9.0 incubation is diluted 10-fold into pH 6.0 buffer and then the same amount of enzyme as before is used to initiate a pH 6.0 assay, the long lag phase of Fig. 1(*a*) is fully re-established. The change recorded on passing from pH 6.0 to pH 9.0 is thus completely reversible.

Fig. 2 shows the results of fluorimetric assays of the enzyme at pH 7.0 with pre-incubation of the enzyme also at pH 7.0. The lag phase is still seen, but is now much shorter than at pH 6.0. Also, dilution of the enzyme to the final assay concentration (2.5 μg/ml) and incubation

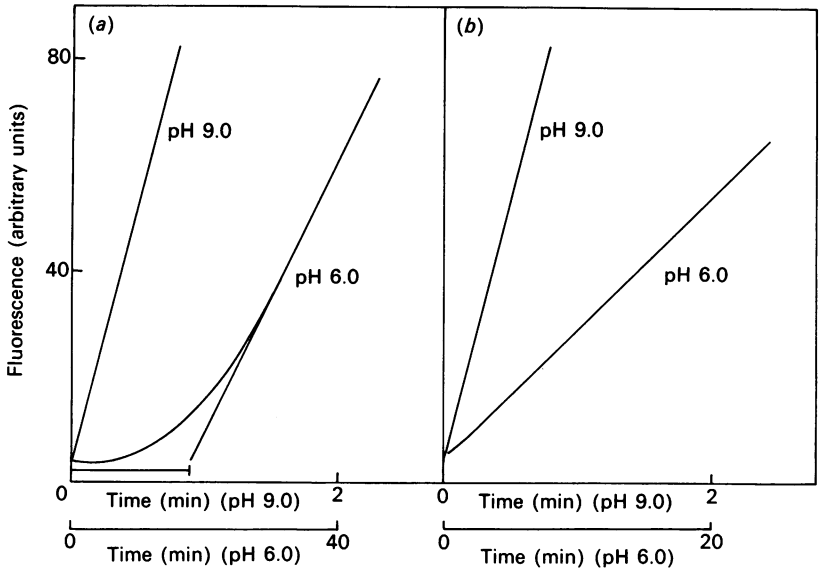

Fig. 1. Fluorimetric assays of UDP-glucose dehydrogenase at 25 °C

Assays were conducted at pH 6.0 in 50 mM-sodium phosphate buffer or at pH 9.0 in 0.1 M-glycine/NaOH buffer with 1 mM-NAD^+ and 0.3 mM-UDP-glucose. A 10 μl portion of UDP-glucose dehydrogenase (1 mg/ml) was used to initiate the reaction. (*a*) Enzyme was maintained for 5 min at 25 °C in the same buffer as used for the assay before initiation of the reaction. The horizontal bar line indicates how the length of lag phases were estimated. (*b*) Enzyme was maintained for 5 min at 25 °C in the alternative buffer before initiating the reaction. For the pH 9.0 assay the enzyme was pre-incubated at pH 6.0 and vice versa.

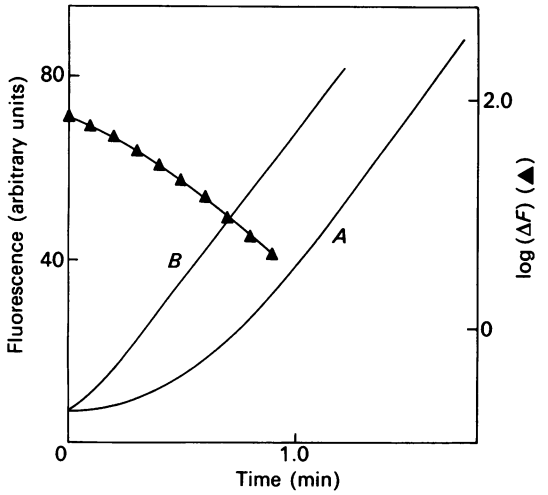

Fig. 2. Fluorimetric assays of UDP-glucose dehydrogenase at 25 °C and pH 7.0

Assays were conducted in 50 mM-sodium phosphate buffer, with 1 mM-NAD^+ and 0.3 mM-UDP-glucose. Curve *A* shows the progress curve after initiation of the reaction with UDP-glucose dehydrogenase (1 mg/ml) maintained in 50 mM-phosphate buffer, pH 7.0. The final enzyme concentration was 2.5 μg/ml. Curve *B* shows the progress curve after initiation of the reaction by addition of 1 mM-NAD^+ and 0.3 mM-UDP-glucose. The enzyme was pre-incubated in buffer at 25 °C at a concentration of 2.5 μg/ml for 2 min before initiation of the reaction. ▲, This curve shows the analysis of curve *A* by the method of Dalziel *et al.* (1978).

for 2 min before the addition of substrate and coenzyme is sufficient almost to abolish the lag. Longer incubation times do not produce any further reduction, and pre-incubation for 2 min at the assay concentration in the presence of either NAD^+ or UDP-glucose, before initiation of reaction with the missing component, does not change the behaviour from that already noted. The behaviour of the enzymes in assays at pH 6.0 and 7.0 is then determined by its concentration immediately before the initiation of the reaction, as well as by the pH of the pre-incubation mixture.

Analysis of the lag phase by the method of Dalziel *et al.* (1978) shows (Fig. 2) that the activation process does not follow simple first-order kinetics. The slope of the plot increases throughout the activation period. Similar, though more markedly curved, plots are obtained on analysis of progress curves obtained from assays done at pH 6.0 and 6.5. This seems to be a definite feature of the activation process.

As the enzyme is normally stored in phosphate buffer, pH 7.0, it is of interest to record the behaviour of assays performed at different pH values, but with the enzyme used to initiate the assay kept at a concentration of 1 mg/ml in 50 mM-sodium phosphate buffer, pH 7.0. Table 1 shows how the length of lag phase and the steady-state rate change. The lag phase is measured as is indicated by the bar line in Fig. 1(*a*). It is evident that the lag phases are very short or even non-existent at alkaline pH values and thus present no problem in routine assays of the enzyme at pH 8.5.

A point of interest is whether the activity of the enzyme as measured in the steady state is concentration-dependent. To test this assays have been conducted at pH 6.0 and pH 9.0 over a wide range of enzyme concentrations and with the use of both spectrophotometric and fluorimetric methods to monitor the reaction. The spectrophotometer assays allow the use of much higher enzyme concentrations than could be used in fluorimetric assays. The results obtained are shown in Fig. 3 and from them it is clear that the specific activity of the enzyme is independent of the enzyme concentration over a 100-fold range at pH 9.0, but is markedly concentration-dependent at pH 6.0 with the activity being lower at higher enzyme concentrations. At pH 9.0 there are of course no lag phases, but at pH 6.0 these are also concentration-dependent. A lag phase of 12 min with 4.3 nM enzyme is shortened to 2 min with 580 nM

Table 1. Assay characteristics of UDP-glucose dehydrogenase as a function of pH at 25 °C

Assays were performed as described in the text with a final enzyme concentration of 2.5 μg/ml. The lag phases were measured as indicated for Fig. 1(*a*) and the rates were those achieved at the end of the lag phase. For the pH range 6.0–8.1 50 mM-sodium phosphate buffers were used and for the pH range 8.5–10.0 100 mM-glycine/NaOH buffers were used.

pH	Lag (min)	Relative rate
6.0	17	3.1
6.5	5	9.6
7.0	0.9	25
7.5	0.25	53
8.1	0.08	84
8.5	0.08	89
9.0	0.01	100
9.5	0	68
10.0	0	18

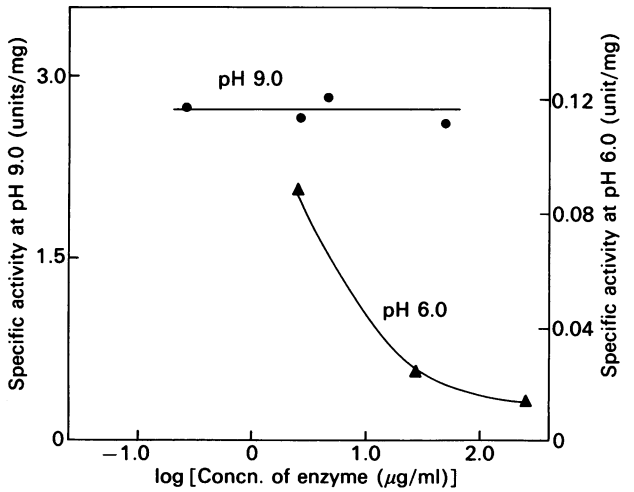

Fig. 3. Variation of the specific activity of the enzyme with enzyme concentration at 25 °C

Assays were conducted at pH 6.0 in 50 mM-sodium phosphate buffer or at pH 9.0 in 100 mM-glycine/NaOH buffer as indicated. The assays were carried out as shown for Fig. 1(*a*). The specific activities were calculated from the rates achieved on completion of the lag phases.

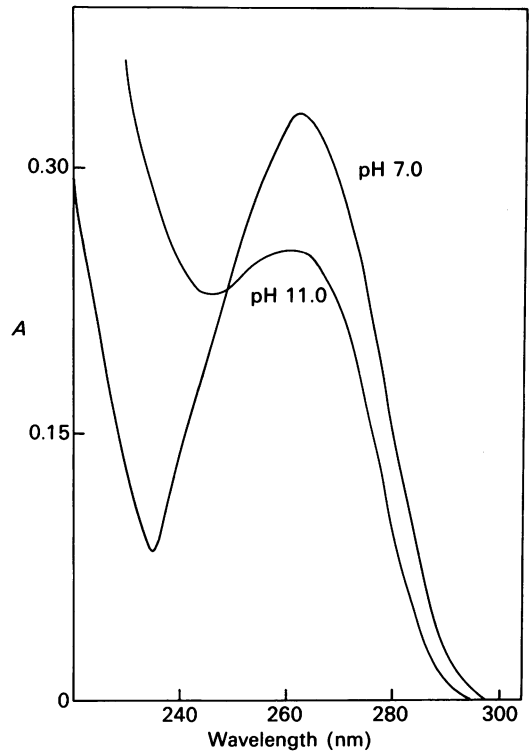

Fig. 4. Absorption spectra of nucleotide material F1

F1 was obtained by boiling UDP-glucose dehydrogenase and purified by ion-exchange chromatography as described in the text. The spectra were taken at identical concentrations at the pH indicated. At pH 2.0 and pH 5.5 spectra identical with that at pH 7.0 were obtained.

enzyme. With 43 nM enzyme both spectrophotometric and fluorimetric methods can be used. Both give essentially the same result with a lag of 5 min. It may be added here that partially purified enzyme (approx. 5% pure) gives the same result in assays at pH 6.0. The feature then appears not to be a result of high purification or to changes in the enzyme on storage.

The concentration-dependence of the lag phase (Fig. 2) and of the specific activity at pH 6.0 (Fig. 3) suggest that the enzyme exists in some form of association–dissociation system. A similar result and conclusion have been obtained with sheep liver mitochondrial aldehyde dehydrogenase (Hart & Dickinson, 1978; Allanson & Dickinson, 1984). Figs. 1, 2 and 3 could be interpreted on the assumption that the aggregate form is inactive but dissociates to the active form on dilution, and that the dissociation occurs slowly at pH 6.0 but much more quickly at pH 9.0. The dependence of specific activity on enzyme concentration at pH 6.0 (Fig. 3) would arise if the dissociation was partial. The equilibrium position would favour the dissociated species and higher activities at lower enzyme concentrations.

The great difficulty with the above hypothesis comes from studies of molecular size. According to the detailed work of Jaenicke *et al.* (1986) the enzyme exists as a stable hexamer of M_r 300000 in the pH range 5.5–7.8 at concentrations greater than 5 μg/ml. High-M_r aggregates of the hexamer occur on storage of the enzyme at pH 5.5, but these are not stable at pH 7.0 and the hexamer is regenerated (Zalitis & Feingold, 1969). The enzyme used here is stored at pH 7.0. Gel-filtration experiments performed in the present study at pH 6.0 and pH 9.0 (mean effluent concentration 200 μg/ml) and gel-electrophoresis experiments at pH 8.8 by the method of Hedrick & Smith (1968) (initial enzyme concentration 500 μg/ml) confirm an M_r in the region 280000–310000.

The evidence that the enzyme does participate in some kind of association–dissociation system comes from experiments where catalysis is occurring, and this may represent a vital difference from the experiments where estimations of M_r have been made. As is shown below, modification of the enzyme by partial reaction of the active-site thiol group with 5-{[(iodoacetamido)ethyl]-amino}naphthalene-1-sulphonic acid dramatically alters the reaction profile in assays at pH 6.0. The formation of reaction intermediates in the catalytic cycle and the sequestering of enzyme in the form of the rate-limiting intermediate might cause similar changes. At the moment it is difficult to give an explanation of the data of Figs. 1, 2 and 3 not involving some form of association–dissociation. Clearly more work is needed.

An alternative hypothesis to that presented above is that there is a tightly bound non-protein inhibitory component that is increasingly dissociated from the enzyme at high dilution and at more alkaline pH values. The enzyme would become active when this species dissociated. As is documented below, enzyme preparations do contain stoichiometric quantities of a non-covalent but tightly bound uridine nucleotide. This finding gives the hypothesis some credence. At this stage, however, it appears that this does not explain the lag phases in assays. Dialysis of the enzyme at pH 9.0 for 24 h at room temperature versus a large excess of buffer would be expected to remove the low-M_r species if it were largely dissociated under these conditions. As shown in Fig. 1(*b*), enzyme kept at pH 9.0 does not show lag phases in assays at pH 6.0, and the 'inhibitor', if that is what the uridine compound is, would be dissociated from the enzyme. Assays of the dialysed enzyme after it has first been returned to pH 6.0 by a short additional dialysis show precisely the same long lags (Fig. 1*a*) that were apparent before the 24 h pH 9.0 dialysis. The effects noted above on changing from pH 6.0 to pH 9.0 are fully reversible even after an extended period at pH 9.0. This seems to suggest that the property is intrinsic to the protein and that the original hypothesis is the more likely.

Attempts have been made to correlate the pH-dependent behaviour of the enzyme in assays (Fig. 1) with some structural feature. Initially fluorescence emission spectra of dilute enzyme solutions (0.3 mg/ml) were taken at pH 9.5 and pH 6.0. No significant differences were detected, however. More encouraging results were obtained on treatment of the enzyme with disulfiram (tetraethylthioperoxydicarbonic diamide). This is a thiol-blocking reagent that reacts extremely rapidly with cytosolic aldehyde dehydrogenase (see, e.g., Dickinson *et al.*, 1981). UDP-glucose dehydrogenase is, of course, an aldehyde dehydrogenase, and the two enzymes operate by mechanisms involving thiohemiacetal and thioester intermediates (Feingold & Franzen, 1981; Dickinson, 1986). In the present work 8 μM enzyme was incubated with 10 μM-disulfiram at pH 6.0, 7.1, 7.7 and 9.0. Assays were initiated at selected times by adding NAD^+ and UDP-glucose. At pH 6.0 the enzyme was completely stable for 5 min. At pH 7.1 it was

inactivated with a half-life of 1 min, and at pH 7.7 the half-life was 15 s. At pH 9.0 it was completely inactivated within the time taken (approx. 5 s) to take the first measurement. Thus there appears to be a correlation between the sensitivity of the enzyme to disulfiram and the activity of the enzyme in assays. The form of the enzyme that is fully active is vulnerable to rapid reaction and inactivation by disulfiram. The inactive form of the enzyme is insensitive to the reagent.

It may be added in connection with the disulfiram inactivations at pH 7.7 that addition of 0.3 mM-NAD^+ or 0.3 mM-UDP-glucose increased the half-life of the inactivations from 15 s to 1 min and 2 min respectively.

Treatment of equimolar concentrations of enzyme with 5-{[(iodoacetamido)ethyl]amino}naphthalene-1-sulphonic acid at pH 8.0 at 30 °C for 45 min should, according to Franzen *et al.* (1980), alkylate an average of 3 sites/hexamer and leave 20–25 % residual activity. The reagent alkylates the active-centre thiol group and introduces a fluorescent probe at that site. In the present work 108 μM subunits was treated with 108 μM reagent, and 35 % activity remained after removal of excess reagent by dialysis. The modified enzyme displayed the expected fluorescence characteristics, but these were the same whether the enzyme was maintained at pH 6.0 or at pH 9.0. Thus no correlation with enzyme assay behaviour and pH is seen at this level. However, the modified enzyme behaves differently in assays. Thus at pH 6.0 (see Fig. 1*a*) the long lag (15 min) is shortened to 1–2 min and the true initial rate is no longer zero but about 30 % of the final steady-state rate. However, the ratio of the final steady-state rates at pH 6.0 and at pH 9.0 is about the same for modified and unmodified enzyme.

Enzyme-bound nucleotide

The purified enzyme preparations exhibit A_{280}/A_{260} values in the range 1.3–1.4, suggesting the possibility of some tightly bound nucleotide-type compound being present. Spectral analysis of the supernatant remaining after boiling the enzyme for 2 min and removal of the denatured enzyme confirms that this is so. The supernatant shows a peak at 262 nm, and on the assumption (see below) that it is a uridine-containing compound 4.5–5.5 mol is liberated/mol of enzyme (i.e. 0.75–0.9 mol/mol of subunit). Other preparations have been treated with $HClO_4$ (0.35 M) with the liberation of 6.0–6.5 mol of nucleotide (1–1.1 mol/mol of subunit). Preliminary analyses with yeast alcohol dehydrogenase and glucose-6-phosphate dehydrogenase established that the compound is not NAD^+ or $NADP^+$. Fluorescence measurements further established that it is not NADH or NADPH. All the compounds mentioned are reasonably stable (80–100 % recovery) to 2 min at 100 °C under the conditions employed. The results, together with the substrate requirements of the enzyme, suggested that the material might alternatively be a uridine-containing compound, and this was later confirmed (see below). However, enzymic tests with UDP-glucose dehydrogenase and NAD^+ established that the released material is not UDP-α-D-glucose. (This compound is completely stable at 100 °C for 2 min at pH 7.0.) Since the enzyme is known to catalyse the oxidation of UDP-α-D-glucohexodialdose (Ridley *et al.*, 1975; Eccleston *et al.*, 1979), the material released cannot be this compound either. It is further established by ion-exchange chromatography and reversed-phase h.p.l.c. (see below) that the material is not UDP-α-D-glucuronate. The compound released is not, then, the normal substrate, intermediate or product of the enzymic reaction.

The non-protein material released by 2 min boiling was examined by ion-exchange on TSK-DEAE-5PW with a phosphate gradient at pH 7.0. Two components were observed. The principle component, F1, comprised 80–85 % and emerged early, close to UMP, UDP-glucose, UDP-mannose, UDP-xylose and UDP-galactose when these were run as markers. Of the compounds tested the sample behaved most like UDP-xylose, but all the compounds ran rather close together. The minor component, F2, emerged late in the gradient in a position close to UDP. UDP-glucuronate emerged only when the gradient was complete, so that both F1 and F2 are quite distinct from this compound. Reversed-phase h.p.l.c. confirmed this finding.

The material released from 80 mg of enzyme by 2 min boiling was subjected to batch ion-exchange chromatography on the same TSK-DEAE-5PW column, but now with an NH_4HCO_3 gradient. The F1 material was bulked together and the F2 material was bulked separately. F1 and F2 were subsequently concentrated by freeze-drying before being dissolved in a small volume of water.

Spectral analysis of F1 at different pH values (Fig. 4) shows that this compound behaves as expected for a uridine-containing compound (Hurlbert, 1957). The spectral characteristics are very different from those exhibited by guanine-, cytosine- or adenine-containing compounds. F2 showed the same spectral characteristics as F1.

Reversed-phase h.p.l.c. analysis of F1 and F2 with 0.5 M-potassium phosphate buffer, pH 6.0, showed that they ran in the region expected for uridine-containing nucleotides. According to Rottlán *et al.* (1986) the uridine nucleotides emerge after the cytosine nucleotides and well before guanine nucleotides, thymine nucleotides and adenine nucleotides. The retention time for F1 was 248 s and that for F2 213 s. The retention times of the other compounds tested were: UMP, 240 s; UDP, 226 s; UDP-glucose, 218 s; UDP-glucuronic acid, 193 s; UDP-mannose, 210 s; UDP-galactose, 220 s; UDP-xylose, 244 s. Again F1 and F2 are not identifiable easily, but F1 behaves more like UDP-xylose than any of the other UDP-sugars tested. The same was true in the TSK-DEAE-5PW ion-exchange chromatography described above. It is noteworthy that UDP-xylose is a powerful inhibitor of the enzyme (Franzen *et al.*, 1983). F2 behaves similarly to UDP-mannose in the reversed-phase h.p.l.c. experiments, but quite differently from it on ion-exchange, so that F2 is evidently not UDP-mannose. Lack of material has meant that no further experiments were made with F2.

Phosphate analysis of F1 by the method of Ames & Dubin (1960) showed that 2.06 mol of phosphate/mol of uridine was released by dry-ashing. In similar experiments with authentic UDP-glucose 1.75 mol of inorganic phosphate was released/mol of uridine. It was concluded on the basis of these results that F1 is a UDP-sugar, a conclusion supported by high-voltage electrophoresis experiments.

In paper electrophoresis at pH 3.5 UMP, UDP and UDP-glucose have the relative mobilities of 0.55, 1.0 and 0.78 and are clearly distinguishable. UDP-mannose and UDP-xylose have the same mobility as UDP-glucose. F1

behaved just like the UDP-sugars in electrophoresis experiments, but after boiling for 10 min in 10 mM-HCl the mobility of the uridine-containing spot changed to that characterstic of UDP, and on boiling for 10 min in 1 M-HCl to that characteristic of UMP. These latter digestions are expected to generate UDP and UMP from UDP-sugars (Dawson *et al.*, 1969), and it appears then that F1 is a UDP-sugar. Unfortunately, at this stage the identity of the sugar is unknown.

An important question is what role the components F1 and F2 play and how they affect enzyme activity. So far there are no real clues about this because attempts to obtain an active apoenzyme have failed. Treatment of enzyme (5.5 mg/ml) at pH 7.0 with increasing amounts of a 15 mg/ml suspension of activated charcoal did not change the A_{280}/A_{260} ratio of the preparation, and subsequent boiling of the treated enzyme released 5 mol of uridine-containing material/mol of enzyme. The catalytic behaviour (Fig. 1) of the enzyme was unaltered by the charcoal treatment, but some enzyme seemed to be lost by binding to the charcoal. In a separate experiment enzyme (6.7 mg/ml) at pH 7.0 was treated with an acid $(NH_4)_2SO_4$ solution so that the mixture was brought to pH 2.9 at an $(NH_4)_2SO_4$ saturation of 65%. This procedure of Warburg & Christian (1938) is often used to resolve flavoproteins into apoenzyme and free flavin. In the present case the protein was precipitated, but could not be redissolved in neutral buffer. The supernatant contained the free prosthetic group as expected. By this treatment 5.5 mol of uridine-containing material/mol of enzyme was liberated, more or less the same as with the heat-denaturation or $HClO_4$ precipitation methods.

The role of the uridine-containing compound is unknown, but the stoichiometry of binding (approx. 1/subunit) shows that it is extremely unlikely that the compound arises from contamination with another enzyme. As the enzyme does not release its intermediate product UDP-α-D-glucohexodialdose in the course of the overall oxidation to UDP-glucuronate it is tempting to suggest that the compound released is in some way related to this intermediate. The fact that the released compound is not a substrate shows that the compound is not UDP-α-D-glucohexodialdose (see above), but this compound is likely to be rather reactive and possibly unstable to boiling. Thus it might be converted into a related species that is not active in the enzyme reaction. Further work is needed to test this idea.

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