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The Binding of Oxidized and Reduced Nicotinamide–Adenine Dinucleotides to Bovine Liver Uridine Diphosphate Glucose Dehydrogenase

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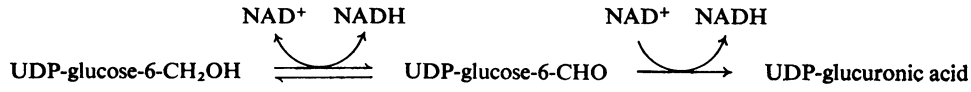
The binding of NAD^+ and NADH to bovine liver UDP-glucose dehydrogenase was studied by using gel-filtration and fluorescence-titration methods. The enzyme bound 0.5mol of NAD^+ and 2mol of NADH/mol of subunit at saturating concentrations of both substrate and product. The dissociation constant for NADH was 4.3μM. The binding of NAD^+ to the enzyme resulted in a small quench of protein fluorescence whereas the binding of NADH resulted in a much larger (60–70%) quench of protein fluorescence. The binding of NADH to the enzyme was pH-dependent. At pH8.1 a biphasic profile was obtained on titrating the enzyme with NADH, whereas at pH8.8 the titration profile was hyperbolic. UDP-xylose, and to a lesser extent UDP-glucuronic acid, lowered the apparent affinity of the enzyme for NADH.

UDP-glucose dehydrogenase (EC 1.1.1.22) catalyses the production of UDP-glucuronic acid from UDP-glucose. The reaction is unusual in that 2mol of NAD^+ are utilized in the overall four-electron oxidation. Nelsestuen & Kirkwood (1971) have proposed the following reaction pathway for bovine liver UDP-glucose dehydrogenase:



The initial two-electron oxidation is apparently reversible whereas the second two-electron oxidation is effectively irreversible.

Zalitis & Feingold (1969), Uram (1971), Huang *et al.* (1971) and P. A. Gainey & C. F. Phelps (unpublished work) have shown the enzyme to be a hexamer with subunit mol.wt. 52000. The enzyme consists of identical or near identical subunits (Gainey *et al.*, 1972). Uram (1971) and Gainey *et al.* (1972) have shown one thiol group/monomer to be essential for enzymic activity.

Franzen *et al.* (1973), by using differential fluorescence and equilibrium dialysis, have reported three NAD^+- and three UDP-glucose-binding sites/hexamer. In the present paper we report results obtained for the binding of NAD^+ and NADH to UDP-glucose dehydrogenase by using both gel-filtration and fluorescence-titration methods.

Experimental

Materials

Chemicals were obtained from the following sources: Norit A (acid-washed) from Kodak Ltd., Kirkby, Lancs., U.K.; bovine serum albumin (fraction V), UDP-xylose and 2-mercaptoethanol from Sigma (London) Chemical Co., London S.W.6, U.K.; [U-^{14}C]NAD^+ (sp. radioactivity, 50–55mCi/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K.; DEAE-cellulose (DE-52, microgranular type) from H. Reeve Angel and Co. Ltd., London E.C.4, U.K.; NAD^+, UDP-glucose and UDP-glucuronic acid from Boehringer Corp. (London) Ltd., London W.5, U.K.; Sephadex G-50 (fine grade) from Pharmacia, Uppsala, Sweden. Dialysis tubing was from Visking Union Carbide, Chicago, Ill., U.S.A., and was boiled for 3–6h with three changes of double-distilled water before use.

Bovine liver UDP-glucose dehydrogenase was purified to a specific activity of 3.3 units/mg by using a modification of the method described by Zalitis & Feingold (1969). Polyacrylamide-gel electrophoresis of the purified enzyme showed the presence of a major protein band corresponding to UDP-glucose

dehydrogenase and a minor protein band corresponding to about 5% impurity. No other dehydrogenase activity was present. The purified enzyme was treated with Norit A in a similar way to that described by Taylor *et al.* (1948). Norit A was added to the enzyme solution to a concentration of 30mg/ml (a threefold excess over protein concentration). After gentle agitation for 10min at 0–4°C the Norit A was removed by suction through a Millipore filter. About 90–100% of the enzyme activity was recovered after this procedure. Protein concentrations were determined from measurements of extinction at 277nm by assuming that a 1mg/ml solution of the enzyme has an extinction of 1.0 (Zalitis & Feingold, 1969). The concentrations of all enzymes are expressed as the concentrations of the subunits by assuming subunit mol.wt. 52000 (Gainey *et al.*, 1972; Zalitis & Feingold, 1969).

Methods

Purification of NADH and NAD$^+$. Commerical NADH was purified by using DEAE-cellulose chromatography (Stinson & Holbrook, 1973). NADH (100–200mg) in 5ml of water was applied to a column (12cm×2.5cm^2) of DEAE-cellulose (bicarbonate form) which had been well washed with water. The column was eluted with a linear gradient of $(NH_4)_2CO_3$, obtained by mixing 500ml of 1M-$(NH_4)_2CO_3$ with 500ml of water. Fractions (20ml) were collected. NADH was eluted in the latter part of the major peak of u.v. absorption at between 0.7M- and 0.9M-$(NH_4)_2CO_3$. Fractions with an E_{340}/E_{260} ratio of 0.44 were combined and freeze-dried or frozen at −10°C. The NADH was redissolved in water just before use and the concentration of the solution determined spectrophotometrically by assuming an ε_{340} of 6.22 litre·mmol^{-1}·cm^{-1}.

NAD$^+$ contains dehydrogenase inhibitors (Babson & Arndt, 1970; Dalziel, 1963). Commerical NAD$^+$ (250mg of the sodium salt) was adsorbed on a column (15cm×2cm^2) of Dowex 1 (Cl^- form) and eluted with 15mM-HCl. The NAD$^+$ eluted as the acid emerged from the column. The eluent containing NAD$^+$ was neutralized with Na_2CO_3 and frozen at −20°C. The frozen NAD$^+$ solution was thawed just before use and the NAD$^+$ concentration determined spectrophotometrically by assuming an ε_{260} of 18.0 litre·mmol^{-1}·cm^{-1}.

Measurement of bound NADH and NAD$^+$ by gel filtration. The coenzyme-binding capacity of the enzyme was measured by the method of Hummel & Dryer (1962). A column (25cm×1cm^2) of Sephadex G-50 was equilibrated with buffer (0.01M-Tris–HCl, pH8.7, containing 1mM-2-mercaptoethanol and 0.16M-NADH). The extinction at 340nm of the eluant was continually monitored on a recorder with a flow-cuvette of 4mm light-path, coupled with a Unicam SP.500 modified as a variable-wavelength split-beam spectrophotometer with a linear output coupled to a chart recorder. When equilibrium was reached (shown by a constant baseline at 340nm of the column eluent) 0.5, 0.25 and 1.0ml samples of buffer were applied to the top of the column at 10min intervals. After the emergence of the last trough corresponding to the 1.0ml buffer calibration, 5.7mg of enzyme in a final volume of 1.0ml of buffer–NADH solution was applied to the column and the column was eluted with the same buffer–NADH solution. The method of Hummel & Dryer (1962) was also used to determine the number of NAD$^+$-binding sites. The equilibrium buffer was 0.01M-Tris–HCl, pH8.0, and contained 0.1mM-NAD$^+$. The column eluent was monitored at 260nm by using a 1mm-light-path flow-cell. The experimental procedure was essentially that described for experiments on the number of NADH-binding sites.

The number of NAD$^+$-binding sites was also investigated by using [U-^{14}C]NAD$^+$. A column (30cm×1cm^2) of Sephadex G-50 was equilibrated with buffer (0.02M-Tris–HCl buffer, pH8.0, containing 2mM-2-mercaptoethanol, 0.909mM-NAD$^+$ and 0.0466μCi of [U-^{14}C]NAD$^+$/ml of buffer). The eluent from the column passed first through a scintillation counter adjusted to measure ^{14}C disintegrations by means of a NE808 flow-head [Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh, U.K.] and then through a 3mm round flow-cell of a Uvicord I photometer (LKB-Producter AB, Stockholm, Sweden) to measure changes in transmitted light at 254nm. ^{14}C radioactivity was counted in an effective volume of about 0.5ml. Both the output from the ratemeter (time-constant 30s) and that of the Uvicord were displayed on a two-channel recorder. When equilibrium was obtained 0.45ml of enzyme (10mg/ml; previously dialysed against 0.02M-Tris–HCl buffer, pH8.0, containing 2mM-2-mercaptoethanol) plus 0.05ml of 9.09mM-NAD$^+$ containing 0.0233μCi of [U-^{14}C]NAD$^+$ was applied to the column. The sample was allowed to drain to bed-height then washed on to the column with 2ml of equilibrium buffer and the column was eluted with the same buffer. The elution of the protein was indicated by an increase in the radioactivity counting rate and a decrease in the transmission at 254nm. In each of the binding experiments the amount of ligand bound to the enzyme was estimated by comparison of the relevant peaks or troughs with the included trough areas corresponding to the buffer calibrations.

NADH and protein fluorescence studies with NADH. Titrations with NADH were performed by using a titrating split-beam differential fluorimeter as described by Holbrook (1972). A split-beam of monochromatic radiation, 280nm for protein fluorescence and 344nm for NADH nucleotide fluorescence, was used to excite fluorescence in two 1cm^2 quartz

cuvettes, one containing UDP-glucose dehydrogenase and the other bovine serum albumin or tryptophan as a reference. Fluorescence was observed at 90° through Kodak-Wratten no. 18A filters (maximum transmission at 335nm) for protein fluorescence and Kodak-Wratten no. 98 filters (maximum transmission at 435nm) for nucleotide fluorescence by using two matched photomultipliers. In the differential mode the signals from each photomultiplier were subtracted by a small analogue computer and after calibrated amplification the difference signal was displayed as protein or nucleotide fluorescence on a chart recorder. The temperature was 21±2°C.

In titration experiments to determine the number of NADH-binding sites, the NADH fluorescence was excited at 385nm to avoid absorption of exciting radiation by relatively high concentrations of NADH. The enzyme was dialysed against the titration buffer (0.01M-Tris–HCl, pH8.8) for 3h before use. Information appropriate to a particular experiment is given in the text.

Stopped-flow fluorescence studies. The fluorescence stopped-flow apparatus was essentially that described by Bagshaw *et al.* (1972). Reactions were studied at room temperature (21±2°C). Further details of individual experiments are given in the text.

Enzyme–NADH difference absorption spectrum. The enzyme–NADH difference spectrum was obtained by using a Unicam SP.1800 double-beam spectrophotometer and four 0.5cm-light-path quartz cuvettes arranged in the tandem position as described by Fischer *et al.* (1969). The temperature was thermostatically controlled at 25°C.

Results

Measurement of bound NADH and NAD$^+$ by gel filtration

The elution profile of the enzyme and the buffer calibrations from the Sephadex G-50 column are shown in Fig. 1. Since the eluent was monitored at 340nm both the peak due to enzyme-bound NADH, and the trough due to depletion of NADH in the near vicinity of the protein were used to calculate the number of NADH-binding sites. Fluorescence-titration experiments with NADH showed that the enzyme was saturated at the NADH concentration (0.16mM) used in these experiments. Some noise was observed on the 'trailing edge' of the peak and may be attributed to 'eddy effects' in the flow-cell caused by the relatively high protein concentrations used. Taking into consideration both peaks and troughs, three experiments showed 2.2, 1.97 and 1.7mol of NADH bound/52000g of enzyme under these conditions.

The protein elution profile in the presence of NAD$^+$ is shown in Fig. 2. The enzyme was eluted from the column with a peak of radioactivity due to bound [U-^{14}C]NAD$^+$. Two experiments, taking both peak and trough into consideration, showed 0.47 and 0.51mol of NAD$^+$ bound/mol of subunit or 2.8–3.1 NAD$^+$-binding sites/hexamer.

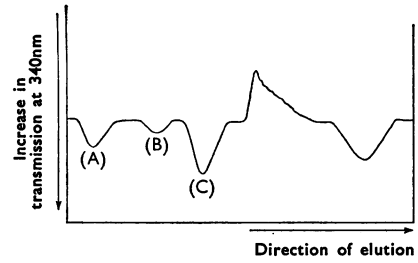


Fig. 1. *Determination of the number of NADH-binding sites on the UDP-glucose dehydrogenase molecule by the Hummel & Dryer (1962) method*

Record of the transmission of 340nm radiation during the gel filtration of 0.097μmol of UDP-glucose dehydrogenase subunits with 160μM-NADH in a final volume of 1ml of 0.01M-Tris–HCl buffer, pH8.7. The column was calibrated by using 0.5 (A), 0.25 (B) and 1.0ml (C) of water as described under 'Methods'. The direction of elution was from left to right.

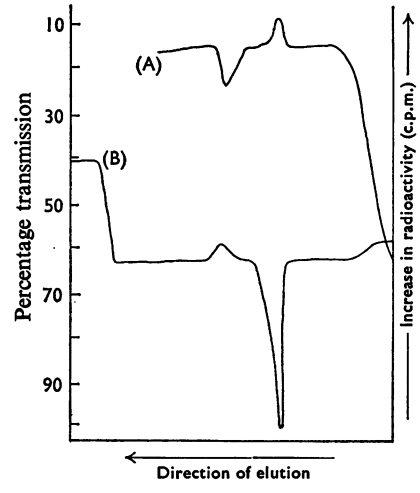


Fig. 2. *Determination of the number of NAD$^+$-binding sites on the UDP-glucose dehydrogenase by using [U-^{14}C]NAD$^+$*

Record of radioactivity (A) and transmission of 260nm radiation (B) during the gel filtration of 0.087μmol of UDP-glucose dehydrogenase subunits and a mixture of NAD$^+$ and [U-^{14}C]NAD$^+$ as described under 'Methods'. The direction of elution was from right to left.

The elution profile of the protein from the column with a tenfold lower NAD^+ concentration and monitoring the column eluent at 260nm gave a value of 0.3mol of NAD^+ binding/mol of subunit, corresponding to 1.8 NAD^+-binding sites/hexamer.

Attempts were also made to determine the number of NAD^+-binding sites by the method of Gerlach *et al.* (1966) and Holbrook & Stinson (1970). However, an increase in absorbance at 325nm, caused by the formation of an enzyme–NAD^+–sulphite complex, was not observed on the addition of Na_2SO_3 to the enzyme in 0.01M-Tris–HCl buffer, pH7.2.

Fluorescence titrations with NADH

Titration of the enzyme with NADH resulted in a quench of both protein and nucleotide fluorescence. By using nucleotide fluorescence it was shown that NADH binding is pH-dependent. NADH binds weakly to the enzyme in 0.01M-sodium phosphate buffer, pH6.5. Fig. 3 shows the biphasic titration profile obtained by using 0.01M-Tris–HCl buffer, pH8.0. A repeat of this experiment with a fivefold decreased titration rate gave the sigmoidal biphasic profile also shown in Fig. 3. On raising the pH to 8.8 a hyperbolic titration profile was obtained (Fig. 3). The total fluorescence amplitude obtained at pH8.8 and 7.2 was 30–40% less than that observed at pH8.0. The quench of protein fluorescence obtained on titration of the enzyme with NADH is shown in Fig. 4. The titration curve is biphasic in profile. The protein fluorescence quench was dependent on the wavelength of the exciting radiation. Maximum quench of protein fluorescence was observed at 285–295nm. There was no change in protein fluorescence quench when the addition of NADH was stopped half-way through a titration. The binding of NADH to the enzyme was ionic-strength-dependent. On lowering the ionic strength of the titration buffer from 0.1 to 0.01M the affinity of the enzyme for NADH was increased about twofold as shown by the more compact biphasic titration profile.

Reaction of NADH with the enzyme in the fluorescence stopped-flow apparatus showed a 30–40% quench of protein fluorescence (Fig. 5). The reaction profile is markedly biphasic.

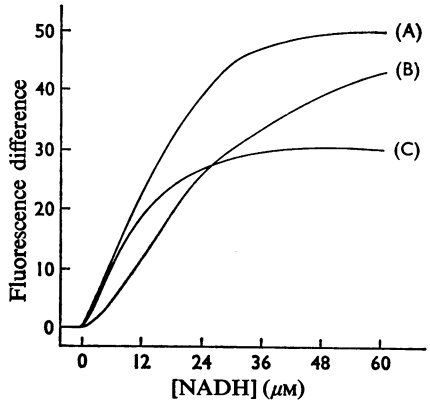


Fig. 3. *Continuous fluorimetric titration of UDP-glucose dehydrogenase with NADH*

NADH (7.23mM) was titrated into 0.014μmol of UDP-glucose dehydrogenase subunits in a final volume of 3ml of 0.01M-Tris–HCl buffer, (A) and (B) pH8.0, and (C) pH8.8. The rate of titration was 1μl and 5μl·min^{-1} for (B) and (A) respectively and 5μl·min^{-1} for (C). Nucleotide fluoresence was excited at 344nm. Amplification was ×10 and the temperature was 20±1°C.

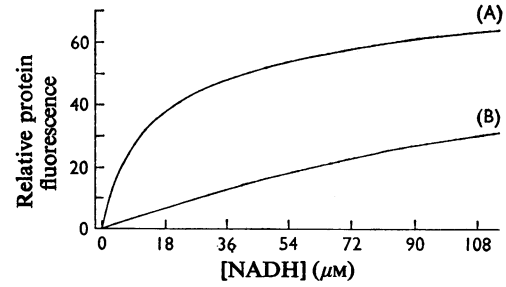


Fig. 4. *Quench of protein fluorescence on titration of UDP-glucose dehydrogenase with NADH*

NADH (5.4mM) was titrated at 5μl/min into (A) 2.3μM-UDP-glucose dehydrogenase solution alone and (B) into 2.3μM-UDP-glucose dehydrogenase solution containing 0.5mM-UDP-xylose. The buffer was 0.01M-Tris–HCl buffer, pH8.2, at 20±1°C. Protein fluorescence was excited at 295nm.

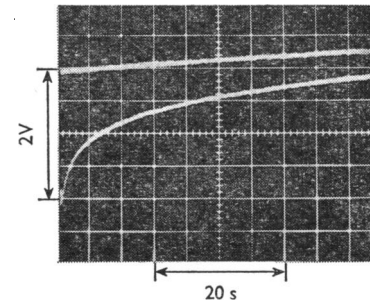


Fig. 5. *Fluorescence stopped-flow record of the reaction of UDP-glucose dehydrogenase with NADH*

UDP-glucose dehydrogenase (6μM subunits) was allowed to react with NADH (20μM) in 0.1M-Tris–HCl buffer, pH8.15. Protein fluorescence was excited at 280nm. The oscilloscope was retriggered after 50s to record the end of the reaction.

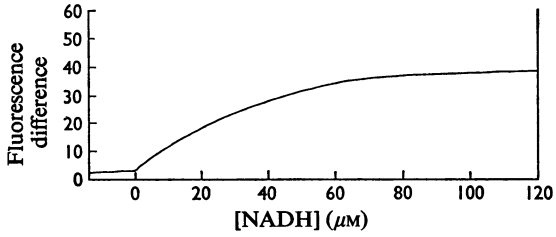


Fig. 6. *Continuous fluorimetric titration of UDP-glucose dehydrogenase to determine the number of NADH-binding sites*

NADH (3.2mM) was continuously titrated, at 5μl/min, into 0.071μmol of UDP-glucose dehydrogenase subunits contained in a final volume of 3ml of 0.01M-Tris–HCl buffer, pH8.8. All values have been corrected for changes in absorption of the exciting radiation (385nm) and the emitted radiation (Wratten 98 filter) owing to the added ligand. The temperature was 20±1°C. Amplification was ×50.

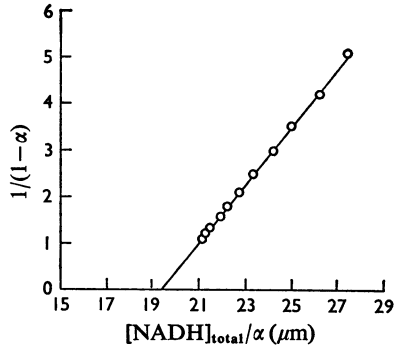


Fig. 7. *Determination of the number of NADH-binding sites on the UDP-glucose dehydrogenase molecule*

The fractional saturation (α) of the NADH-binding sites was measured from the quench of nucleotide fluorescence when NADH (3.2mM) was continuously titrated, at 5μl/min, into 24.4μM-UDP-glucose dehydrogenase in 0.01M-Tris–HCl buffer, pH8.8, at 21±1°C. The protein concentration is calculated by assuming subunit mol.wt. 52000. The concentration of the NADH-binding sites was the intercept with the $[NADH]_{total}/\alpha$ axis when $1/(1-\alpha)=0$. The dissociation constant was the reciprocal slope of the graph.

Titration of the enzyme with NADH to give a 50% quench of protein fluorescence followed by the manual addition of UDP-xylose resulted in a decrease of the protein fluorescence quench. This quench of protein fluorescence could be increased again to the initial value (50%) by the addition of excess of NADH. The presence of UDP-glucuronic acid (0.1mM) also lowered the affinity of the enzyme for NADH approximately twofold at pH8.8. Reaction of NADH with the enzyme–UDP–xylose complex measured by using fluorescence stopped-flow techniques also showed a decrease in the affinity of the enzyme–UDP–xylose complex for NADH.

The quench of nucleotide fluorescence obtained in experiments to determine the number of NADH-binding sites is shown in Fig. 6. A linear relationship was observed between the nucleotide fluorescence (F) and the fraction of coenzyme sites occupied by NADH (α). A plot of $1/(1-\alpha)$ against $[NADH]/\alpha_{total}$ was linear (Fig. 7). The dissociation constant given by the reciprocal slope was 4.3μM. The concentration of NADH-binding sites (E_0), given by the extrapolated intercept of the graph with the $[NADH]_{total}/\alpha$ axis, was 51.1μM. The enzyme concentration (monomer) was 24.4μM. This corresponds to 2.09 NADH-binding sites/52000g of enzyme. The sum (55.4μM) of the dissociation constant (K_d) and of the concentration of NADH-binding sites (E_0) determined from Fig. 7 is in good agreement with that observed experimentally by using the initial-tangent method described by Velick (1958).

Titration of the enzyme with NAD^+ gave a 5–10% quench of protein fluorescence with exciting radiation at 295nm. At 305 and 285nm the observed quench was smaller.

Enzyme–NADH difference spectra

Addition of increasing amounts of NADH to the enzyme did not result in a significant difference spectrum.

Discussion

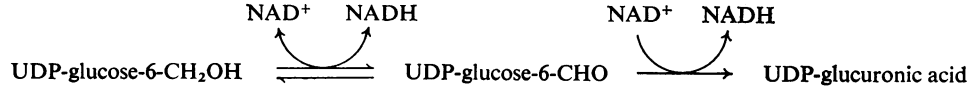
Franzen *et al.* (1973) used equilibrium dialysis and differential fluorescence to study the binding of NAD^+ to UDP-glucose dehydrogenase. At saturation the hexameric enzyme has only three NAD^+-binding sites. UDP-glucose dehydrogenase from bovine liver has been shown to contain six apparently identical, or near identical, subunits. The subunit molecular weight is about 52000 (Uram, 1971; Huang *et al.*, 1971; Gainey *et al.*, 1972). Thus by comparison with many other dehydrogenases (Wallis & Holbrook, 1973; Holbrook & Stinson, 1970) it would appear that UDP-glucose dehydrogenase is unusual in not having one NAD^+-binding site/monomeric subunit. Holbrook & Gutfreund (1973) have stressed the necessity of using the pure coenzyme when investigating the binding of NAD^+ to dehydrogenases. Commercial NAD^+ contains dehydrogenase inhibitors (Babson & Arndt, 1970; Dalziel, 1963). Since UDP-glucose dehydrogenase has a much greater affinity for NADH than NAD^+ care has to be taken to ensure that the NAD^+ is pure and free of NADH or NADH-like adducts.

The binding of NAD^+ to UDP-glucose dehydrogenase was therefore re-investigated with purified

coenzyme. The enzyme was treated with charcoal to remove any tightly bound contaminants (NADH-X). The results obtained by the method of Hummel & Dryer (1962) are in good agreement with those obtained by Franzen *et al.* (1973) who used equilibrium dialysis and differential fluorescence. At lower (0.1mM), and presumably unsaturated, concentrations of coenzyme 1.8mol of NAD^+ are bound/mol of hexamer; with 1mM-NAD^+, only 2.9mol of NAD^+ are bound/mol of hexamer. If it is assumed that the enzyme is near-saturated at this concentration of NAD^+, then it would appear that UDP-glucose dehydrogenase has only three NAD^+ binding sites/hexamer, and also that the relatively unusual NAD^+-binding capacity of this dehydrogenase observed by Franzen *et al.* (1973) is not an artifact caused by coenzyme impurities or by tightly bound enzyme contaminants.

The binding of NADH to UDP-glucose dehydrogenase was facilitated by the greater affinity of the enzyme for NADH compared with NAD^+. Fluorescence titration has shown that UDP-glucose dehydrogenase binds 2mol of NADH/mol of monomer. This result has been confirmed by the method of Hummel & Dryer (1962). The NADH-binding capacity is an unusual feature of UDP-glucose dehydrogenase. A general feature of other dehydrogenases is their ability to bind 1mol of NADH/mol of monomer (Holbrook *et al.*, 1972; Pfleiderer & Auricchio, 1964), although Jallon & Iwatsubo (1971) and Koberstein & Sund (1971) have claimed that 2mol of NADH are bound to 56000g of bovine liver glutamate dehydrogenase in the presence of either GTP, L-glutamate or Mg^{2+}.

Nelsestuen & Kirkwood (1971) have shown, by using the postulated UDP-glucose-6-CHO intermediate, that the overall reaction may be represented by:



The first two-electron oxidation is apparently reversible whereas the second two-electron oxidation is not reversible.

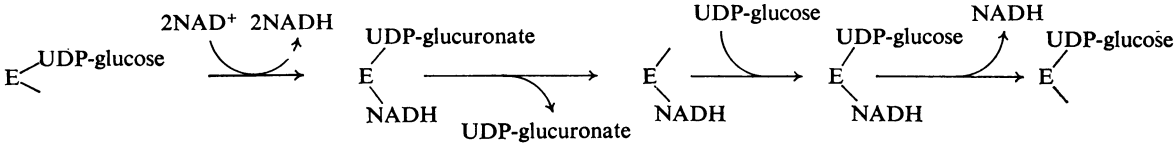
The discrepancy between the numbers of NAD^+- and NADH-binding sites presents several obvious difficulties when considering the reaction pathway postulated above. Several possibilities exist to explain the binding of only 0.5mol of NAD^+ and 2mol of NADH/mol of subunit. First, the binding of NAD^+ to the enzyme may exhibit negative co-operativity; however, the binding data of Franzen *et al.* (1973) suggest identical non-interacting NAD^+-binding sites. Secondly, the conformation of the enzyme which binds NADH may be distinct from that binding NAD^+. Difference spectra, however, suggest that the binding of NADH to the enzyme is not accompanied by a conformational change. Thirdly, it is possible that the initial turnover of the enzyme results in the appearance of further NAD^+-binding sites which because of polypeptide chain folding or symmetry arrangements are unable to bind NAD^+ in the native state.

The biphasic titration curve shown in Fig. 3(*a*) suggests two or more distinct classes of NADH-binding sites. This is in accordance with the reaction mechanism suggested by Nelsestuen & Kirkwood (1971). Several models may be proposed to explain this observation. The first involves two classes of chemically or structurally distinct sites resulting in intrinsically different binding sites. If the binding sites are on chemically identical subunits (Uram, 1971; Gainey *et al.*, 1972), folding of the polypeptide chain or symmetry arrangements can still result in intrinsically different binding sites. The second possibility is that the intrinsically identical binding sites and the structure of the ligand result in the initially liganded sites interfering with the binding to subsequent sites. This could be a steric or a charge-repulsion phenomenon. The third possibility is that the binding of the initial ligand molecules results in a protein conformational change which decreases the affinity for the remaining sites. Winlund & Chamberlin (1970) have reported non-identical binding sites in the binding of CTP to aspartyl transcarbamylase.

The difference in total fluorescence amplitude observed by using nucleotide fluorescence and pH8.1 and 7.2 buffers indicates a pH-dependent conformational change. Evidence for this may be seen in the NADH-titration profiles at different pH values (Figs. 3*a* and 3*c*). At pH8.1 the titration curve is biphasic whereas at pH8.8 a hyperbolic titration curve is obtained. Further, the sigmoidal titration profile obtained at pH8.1 suggests co-operative binding of NADH to the enzyme at this pH value. Titration at pH8.8 did not show such co-operative effects. Assuming that the enzyme subunits are identical, the linear plot of $1/(1-\alpha)$ against $[NADH]_{total}/\alpha$ is further evidence that the NADH-binding sites do not interact at this pH (Holbrook, 1972). Similar non-co-operative binding was observed by Franzen *et al.* (1973) with NAD^+ at this pH value.

The presence of UDP-xylose lowers the affinity of the enzyme for NADH (Fig. 4). The observation that the addition of UDP-xylose to the enzyme, previously titrated to 50%–60% quench of protein fluorescence with NADH, resulted in a decrease of the protein

fluorescence quench indicates that UDP-xylose binds to the enzyme–NADH complex, and, further, that the binding of UDP-xylose results in the release of bound NADH. If UDP-xylose acts as a substrate analogue of UDP-glucose (Gainey *et al.*, 1972), then the apparent decrease in affinity of the enzyme for NADH may be explained as follows: at the end of the first turnover of the enzyme NADH and UDP-glucuronic acid are bound to the enzyme. UDP-glucuronic acid, because of its lower affinity for the enzyme (owing perhaps to its negative charge), presumably dissociates from the enzyme before NADH. Because of the great difference in affinity of the enzyme for NAD^+ and NADH it is necessary for the NADH to be removed before the next NAD^+ binds. It is possible that the substrate, UDP-glucose, lowers the affinity of the enzyme for NADH, as can be seen by the effect of the substrate analogue UDP-xylose. The above mechanism is represented diagramatically below:



The effect of UDP-xylose on the quench of protein fluorescence obtained with NADH (Fig. 4) suggests that the binding of UDP-xylose causes the dissociation of bound NADH from the enzyme. An alternative explanation, although a less convincing one, is that the presence of UDP-xylose may cause the dissociation of the oligomeric protein, with bound NADH, into lower-molecular-weight subunits, as has been observed on the addition on NADPH to glutamate dehydrogenase from *Neurospora crassa* (Gore *et al.*, 1972). Centrifugation studies with the enzyme in the presence of both NAD^+ and UDP-xylose (P. A. Gainey & C. F. Phelps, unpublished work), however, show no change in the molecular weight when compared with the native enzyme.

The observation that both UDP-xylose and UDP-glucuronic acid appear to lower the affinity of the enzyme for NADH may be taken as further evidence that UDP-xylose acts as a substrate analogue of UDP-glucose (Gainey *et al.*, 1972). Neufeld & Hall (1965) have shown that UDP-glucuronic acid is a competitive inhibitor of both the bovine liver and the pea-seedling enzyme, with respect to UDP-glucose.

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