UDP-GLUCOSE DEHYDROGENASE: SUBSTRATE BINDING STOICHIOMETRY AND AFFINITY

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SUMMARY: The direct binding of UDP-glucose and NAD^+ to bovine liver UDP-glucose dehydrogenase has been measured by equilibrium dialysis and differential fluorescence. At saturation the hexameric enzyme binds only three molecules each of UDP-glucose and NAD^+. The binding of NAD^+ is virtually characteristic of that for noninteracting identical sites with a binding constant of about 0.47×10^4. UDP-Glucose, however, binds more avidly than NAD^+ and exhibits negative cooperativity characterized by unrestricted Adair constants of 16.1, 3.7, and 0.37, all $\times 10^4$.

UDP-Glucose dehydrogenase from beef liver has been shown to contain six apparently identical 52,000 dalton subunits (1-4). The oxidation of the terminal hydroxyl of the glucose moiety is a two-stage process involving an overall transfer of four electrons (5). Early kinetic analyses indicated the classical Michaelis-Menten response of the catalytic process to substrate concentration (6,7), though later investigation indicated more complicated cooperative effects in the presence of the allosteric effector, UDP-xylose (8). This report presents evidence regarding the stoichiometry and stability of the enzyme complexes with the substrates UDP-glucose and NAD^+.

MATERIALS. The enzyme was prepared according to the procedure of Zalitas and Feingold (9). Preparations used for binding studies had specific activities, as defined in the previous reference, ranging from 2.5 to 3.0. Non-radiolabeled UDP-glucose and NAD^+ were obtained from Sigma Chemical Co., while UDP-glucose-^{14}C, 227 mCi/mmole, and NAD^+-carbonyl-^{14}C, 50 mCi/mmole, were obtained from New England Nuclear and Amersham/Searle Corp., respectively. Paper chromatographic analysis showed that the UDP-glucose-^{14}C had a radiochemical purity of 85%. Appropriate corrections for the non UDP-glucose-^{14}C

impurity were applied to all binding data, assuming that such material, probably either glucose or glucose-1-phosphate, was not bound to the enzyme.

Binding by Equilibrium Dialysis. Dialyses were carried out in cells similar to those described by Englund, *et al* (10). Each chamber, having a total empty volume of 30 µliters, was loaded with 20 µliters of either enzyme-buffer solution or ligand-buffer solution. Two 1 mm diameter glass beads were introduced into each chamber before assembly of the cell. Prestretched Visking tubing was employed to partition the dialysis chambers. After assembly and loading, the cells were mounted on a rotor, turning at 6 rpm, and allowed to equilibrate for six hours at 0-1°. Duplicate 5 µliter aliquots were then withdrawn from each chamber and assayed for radioactivity content by scintillation counting. The scintillation fluid, 10 ml, was composed of 1 part Triton X-100 and 4 parts toluene-Omnifluor, New England Nuclear. Retention of data for binding ratio calculations was based on the extent of recovery, 100 ± 2%, of radioactivity added to the dialysis cell and the agreement between aliquots taken from the same dialysis chamber. The specific activity of the added ligand was higher in the low ligand concentration ranges than in the high ligand concentration ranges to assure the development of significant radioactivity differences between the two chambers of the dialysis cells. Reversibility was checked by adding ligand to the chamber containing enzyme.

Binding by Differential Fluorescence. The binding of NAD^+ was assessed fluorometrically by observing the quenching of the enzyme fluorescence according to the difference method of Franzen, *et al.*, which self corrects for optical inner filter effects (11). A rectangularly shaped cell was used which thus reduced the optical path length of the incident radiation, 285 nm, through regions of the cell not viewed by the photomultiplier receiving the emitted radiation, 330 nm. The differential fluorescence measurements were made in a temperature controlled cell at 23°, while the equilibrium dialysis measurements of NAD^+ and UDPG binding were made at 0°.

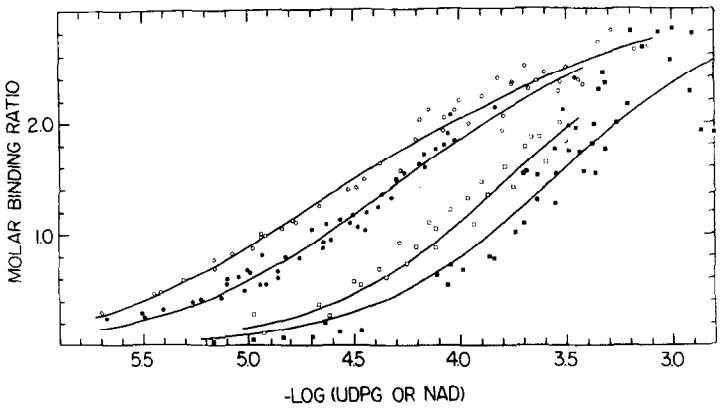

Figure 1. Substrate saturation of UDP-glucose dehydrogenase. Unfilled and filled circles represent binding ratios collected by two different investigators (see text) for UDP-glucose as ligand. Unfilled squares represent fractional saturations (x 3) for NAD^+ as determined by differential fluorescence. Filled squares correspond to binding ratios for NAD^+ as determined by equilibrium dialysis.

Data Analysis. The binding data for UDP-glucose were fitted by the Adair equation according to the computational scheme of Cornish-Bowden and Koshland (12). The necessary computer program was obtained from Professor Koshland and slightly modified for direct analysis of a three site system and for operation on the PDP-11 computer belonging to this department.

The saturation of UDP-glucose dehydrogenase with its substrates is portrayed in Figure 1. The two sets of data for UDP-glucose binding were obtained under identical conditions by two different investigators working on the same enzyme preparation, but at different periods in time. The lower of the two data sets represents measurements made about three months after the first set. The real discrepancy may reflect an aging process, however, the specific activity of the enzyme did not change over this period. Although there exists an unexplained difference in these two UDP-glucose binding isotherms, the essential conclusions to be drawn about the enzyme system are alike in each case. The two solid curves for the UDP-glucose binding data represent the "best fit" Adair functions for a three site system. Notice that saturation is achieved at a binding ratio of 3.0. Table I sum-

Table I. Binding Parameters According to the General Ligand-Induced Model for the Interaction of UDP-Glucose Dehydrogenase with UDP-Glucose and NAD^+.

	UDPG		NAD^+	
Parameter	Data Set 1	Data Set 2	Eq. Dial.	Diff. Fluor.
Ψ_1[a]	16.08 ± 2.85	8.19 ± 1.07	Not Applicable	
Ψ_2	58.73 ± 6.58	15.60 ± 1.68	"	"
Ψ_3	2.14 ± 2.95	5.52 ± 1.38	"	"
R.M.S. Fit	0.110	0.099	"	"
K_1[a]	16.08	8.19	0.58[b]	0.36[b]
K_2	3.65	1.90	NA	NA
K_3	0.365	0.354	NA	NA
k_1[c]	5.36	2.73	NA	NA
k_{12}	3.65	1.90	NA	NA
k_{123}	1.095	1.06	NA	NA

[a] As defined by Cornish-Bowden and Koshland, $\Psi i = \prod_{j=i}^{i} K_j$, where K_j is the macroscopic association constant for the association of the jth ligand to all macromolecular species containing j-1 ligands. These and all other binding constants are reported in terms of $M^{-1} \times 10^4$.

[b] These values were chosen simply by inspection of the NAD^+ binding data of Fig. 1. They correspond to the reciprocal of the NAD^+ concentration at which the enzyme was estimated to be half saturated.

[c] These intrinsic constants, obtained from the K_i values, pertain to the model in which the three empty sites of the unliganded enzyme are identical and are described by k_1 ($=K_1/3$), and the two empty sites of the monoliganded enzyme are identical and are described by k_{12} ($=K_2$), and the one empty site on the biliganded enzyme is described by k_{123} ($= 3K_3$).

marizes the binding constant values that correspond to the isotherms of Figure 1. The relative values of the Adair constants indicate apparent negative cooperativity. This feature is also discernible by inspection of the breadth of the isotherms of Figure 1.

The potential for binding NAD^+, as depicted in Figure 1, is substantially reduced relative to that of UDP-glucose. The binding functions as de-

termined by differential fluorescence and by equilibrium dialysis are clearly not superimposable, though they appear to be of the same form. The shift along the abscissa may result from the different temperatures employed in these two experimental approaches. The stoichiometry of NAD^+ binding at saturation is not as certain as for UDP-glucose binding. The weaker association necessitated the use of high ligand concentrations which introduced unavoidably large experimental errors in the equilibrium dialysis measurements. Even at the largest NAD^+ concentrations used, however, we did not obtain binding ratios greater than three. The differential fluorescence method actually yields fractional saturation values rather than binding ratios (11). These fractional saturations have been multiplied by 3.0 for presentation in Fig. 1. The curves drawn through the NAD^+ binding points of Fig. 1 are simple Langmuir type absorption isotherms based on dissociation constants estimated from the approximate half saturation concentration of NAD^+ for the equilibrium dialysis and differential fluorescence generated points. The imprecision in these measurements makes it impossible to detect deviations from one class of sites behavior.

DISCUSSION. From what is known about the subunit nature of bovine liver UDP-glucose dehydrogenase, it was first suspected that six catalytic sites would exist in the native hexamic form of the enzyme (1-4). It is striking that only three such centers are accounted for by the data set forth here. This result immediately suggests that the binding sites are located in the interfaces between pairs of subunits, i.e. the native enzyme is composed of three dimers. In view of the sequential nature of the two stage oxidation (5), it is not surprising that the two substrates bind in equimolar amounts rather than in a two to one ratio in favor of NAD^+ over UDP-glucose.

The kinetic Hill coefficient of 2.3 for UDP-xylose interaction with the enzyme is also not inconsistent with the stoichiometry obtained by us for UDP-glucose and NAD^+ binding (8). This inferred equivalence of UDP-xylose binding stoichiometry with UDP-glucose binding stoichiometry could be viewed as an-

other bit of evidence for the contention that UDP-xylose is a true competitive inhibitor which introduces homotropic cooperative effects rather than being an allosteric effector (4). Additional binding studies with UDP-xylose now under way will have direct bearing on this question. The additional assertion by Gainey, *et al.*, that an ordered addition of substrates to the enzyme, with NAD^+ being the leading ligand must be carefully scrutinized in consideration of the highly preferred affinity of the enzyme for UDP-glucose demonstrated here (4).

The dampening of the successive stages of UDP-glucose binding that is apparent in Fig. 1 and in the Adair constants of Table I is considered by us to indicate negative cooperativity between sites. The corresponding intrinsic or microconstants are given in the last three rows of Table I. The strong evidence for subunit identity (1-4), leads us to prefer this explanation for the negative deviations from a hyperbolic saturation response over the postulation of classes of independent sites. No attempt was made to fit the data to any more restrictive case than the general Adair formulation. In any event a Monod-Wyman-Changeux model for UDP-glucose dehydrogenase is ruled out (13). Interestingly, the analysis of the binding data by the four site form of the Cornish-Bowden-Koshland program yields Adair constants which are indistinguishable from those of Table I, while the corresponding constant for the fourth site becomes vanishingly small. The fact that UDP-glucose negative cooperative effects have not been heretofore detected is not surprising since the earlier workers did not carefully investigate the dilute concentration range in which this behavior would be expected (6-8). On the other hand, it could be argued that this negative cooperativity behavior observed at 0-1° is inoperative at the high temperatures normally used in enzyme assays.

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