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UDPGLUCOSE DEHYDROGENASE

KINETICS AND THEIR MECHANISTIC IMPLICATIONS *

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Summary

Initial velocity and product inhibition studies were carried out on UDP-glucose dehydrogenase (UDPglucose: NAD^+ 6-oxidoreductase, EC 1.1.1.22) from beef liver to determine if the kinetics of the reaction are compatible with the established mechanism. An intersecting initial velocity pattern was observed with NAD^+ as the variable substrate and UDPG as the changing fixed substrate. UDPglucuronic acid gave competitive inhibition of UDPG and non-competitive inhibition of NAD^+.

Inhibition by NADH gave complex patterns. Lineweaver-Burk plots of $1/v$ versus $1/NAD^+$ at varied levels of NADH gave highly non-linear curves. At levels of NAD^+ below 0.05 mM, non-competitive inhibition patterns were observed giving parabolic curves. Extrapolation to saturation with NAD^+ showed NADH gave linear uncompetitive inhibition of UDPG if NAD^+ was saturating. However, at levels of NAD^+ above 0.10 mM, NADH became a competitive inhibitor of NAD^+ (parabolic curves) and when NAD^+ was saturating NADH gave no inhibition of UDPG. NADH was non-competitive versus UDPG when NAD^+ was not saturating.

These results are compatible with a mechanism in which UDPG binds first, followed by NAD^+, which is reduced and released. A second mol of NAD^+ is then bound, reduced, and released. The irreversible step in the reaction must occur after the release of the second mol of NADH but before the release of UDPglucuronic acid. This is apparently caused by the hydrolysis of a thiol ester between UDPglucoronic acid and the essential thiol group of the enzyme. Examination of rate equations indicated that this hydrolysis is the rate-limiting

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Abbreviations: UDPGlcUA, UDPglucuronic acid: UDPGlc-6-CHO, UDP-α-D-gluco-hexodialdose.

step in the overall reaction. The discontinuity in the velocities observed at high NAD^+ concentrations is apparently caused by the binding of NAD^+ in the active site after the release of the second mol of NADH, eliminating the NADH inhibition when NAD^+ becomes saturating.

Introduction

The formation of UDPGlcUA from UDPG is catalyzed by UDPglucose dehydrogenase (UDPglucose: NAD^+ 6-oxidoreductase, EC 1.1.1.22) in an irreversible reaction in which 2 mol of NAD^+ are converted to NADH [1,2]. The reaction has been demonstrated to involve the reversible formation of UDPGlc-6-CHO from UDPG, followed by the irreversible formation of the corresponding acid [3]. The intermediate aldehyde remains tightly bound to the enzyme surface during the course of the reaction [1].

There is considerable evidence that this enzyme possesses an essential thiol group [4—7] and both the overall irreversibility of the reaction and the tight binding of the aldehyde intermediate can be rationalized by invoking its function. If the essential thiol group functioned to form a thiohemiacetal with the intermediate aldehyde, then the strong binding of the aldehyde is explained. In addition, it is well established that thiol esters have large negative free energies of hydrolysis [9] and the hydrolysis of the resulting thiol ester would account for the overall irreversibility of the reaction. Such a mechanism has been proposed as being general for aldehyde dehydrogenases [10], has been verified in the case of glyceraldehyde-3-phosphate dehydrogenase [11] and has recently been demonstrated in the case of the present enzyme [8].

The purpose of the present work was to determine if the kinetics of the UDPglucose dehydrogenase reaction are compatible with the proposed mechanism. Some inhibition studies have been reported previously. UDPGlcUA has been demonstrated to be competitive with UDPG [2,12,13]. UDPGlcUA is non-competitive with NAD^+ [2,11] and NADH is uncompetitive with UDPG in the case of enzyme from *Escherichia coli* [12]. NADH is reported to be competitive with NAD^+ [2,13,14].

Materials and Methods

Enzymes and chemicals. UDPG dehydrogenase was purified from bovine liver to a specific activity of 0.8 unit/mg by the method of Straw [5] with the exception that the extraction solution and the buffers, other than that used in the ethanol fractionation, contained 2 mM EDTA and 10 mM β-mercaptoethanol. UDPG and UDPGlcUA were obtained from Sigma and NAD^+ and NADH from P and L Biochemicals.

Enzyme assays. All rate measurements were performed at 37°C in a total volume of 0.5 ml. Glycine/NaOH buffer (38 μmol, pH 8.7), 0.5 μmol of neutralized cysteine · HCl and $6 \cdot 10^{-3}$ unit of UDPG dehydrogenase were incubated for 1 min, at which point a mixture of the substrates and products in distilled water (adjusted to pH 8.0) were added to start the reaction. One unit of enzyme is defined as that amount which will produce 1 μmol of UDP-

GlcUA per min under the above assay conditions and in the presence of 1 mM NAD^+ and 1 mM UDPG. All reaction velocities are expressed as μM NADH produced per min for the stated reaction mixture.

All reaction rates were measured spectrophotometrically by following NADH production at 340 nm using a General Medical and Atomic Energy Commission (GEMSAEC) autoanalyzer (Electro-Nucleonics, Fairfield, N.J.). The GEMSAEC consists of a combination centrifuge, spectrophotometer and computer which carries out fifteen assays simultaneously and provides a numerical printout of the absorbance versus time. For this work readings were taken every 15 s for 5 min. An extinction coefficient of $6.3 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ at 340 nm was assumed for NADH [15].

Treatment of kinetic data. Through the use of the theory and nomenclature described by Cleland [16—18], initial velocity and product inhibition studies were used to obtain information regarding the sequence of addition of substrates and release of products from UDPG dehydrogenase during catalysis. Rate equations and inhibition patterns were determined for ter-ter and bi-uni-uni-bi ordered or random mechanisms both with and without separate rate constants for the hydrolysis of a thiol ester in the mechanism, using equations and techniques described by Plowman [19].

Initial estimates of kinetic parameters were obtained by least squares plots followed by replots to isolate individual terms in the equation, and then solution of the terms for numerical values of parameters. These estimates were improved by a program involving a Newton-Raphson iteration scheme [20] that fitted all of the parameters simultaneously to the data. Curves in all figures were drawn by linear or quadratic least squares programs.

Results

Initial velocity studies. Initial velocity studies were carried out as a function of NAD^+ and UDPG concentrations. The enzyme preparation was shown to be free of inhibitors by demonstrating that enzyme concentration multiplied by time equalled NADH production for 5, 10, 20 and 40 μl of enzyme with 0.25 mM NAD^+ and 0.10 mM UDPG. Representative initial velocity data are shown in Fig. 1. The intersecting pattern is characteristic of either a random or an ordered sequential mechanism. In Table I the kinetic parameter terms which can be evaluated from initial velocity studies alone are listed.

NADH inhibition. Of particular interest in determining the mechanism are the patterns of NADH inhibition. Because NAD^+ combines at two distinct points in the reaction sequence, it would not be surprising to find parabolic inhibition by NADH. Because inhibition by NADH has been shown to be competitive only for a narrow range of conditions [2,13,14], and because the data in some cases appear slightly parabolic [2,13], inhibition by NADH was examined thoroughly. Webb [21] has shown that if a reaction product is an inhibitor and its K_i is small, the reaction rate may drop very rapidly after the reaction has started. Use of the GEMSAEC autoanalyzer allowed both rapid, reproducible mixing and measurement of small variations in absorbance (0.0002). Figs. 2a and 2b show plots with NAD^+ as the variable substrate and NADH as the changing fixed inhibitor. In Fig. 2a, NAD^+ concentration was

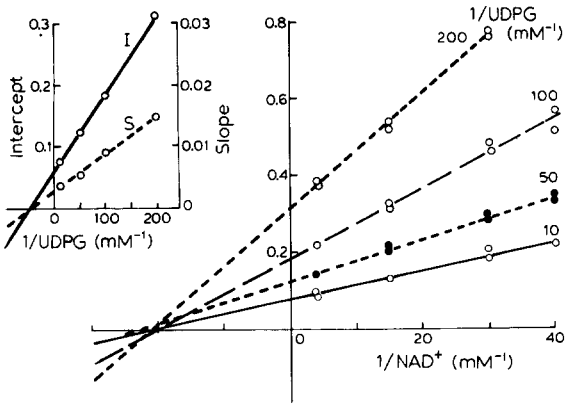


Fig. 1. Initial velocity of UDPG dehydrogenase activity with NAD^+ as the variable substrate and UDPG as the changing fixed substrate. The assay is described under Materials and Methods. The reciprocal of initial velocity of NADH formation, in μM per min, is plotted as a function of the reciprocal of the concentration of NAD^+, in mM, in the primary plot. The slopes and intercepts are replotted as a function of the reciprocal of UDPG concentration in mM in the secondary plot.

varied from 0.1 to 2.0 mM. The pattern shows parabolic competitive inhibition. Similar results were achieved at 0.05, 0.025, and 0.0167 mM UDPG. In Fig. 2b, NAD^+ concentration was varied from 0.01 to 0.05 mM. The pattern indicates parabolic non-competitive inhibition. Similar results were achieved at UDPG concentrations of 0.05, 0.025, and 0.0167 mM.

TABLE I

KINETIC PARAMETERS OF UDPG DEHYDROGENASE FROM INITIAL VELOCITY STUDIES

Values were calculated from replots in Fig. 1 based on Eqn. 1, where product concentrations are all zero. Calculations were done using the method of Elmore et al. [33].

$V = 18.9 \pm 1.9$ μM/min (for the assay conditions)

$K_{NAD_1} + K_{NAD_2} + K_{NAD_3} = 49.9 \pm 7.9$ μM

$K_{UDGP} = 24.6 \pm 3.7$ μM

$K_{iUDGP} * K_{NAD_1} = 1191 \pm 302$ μM^2

where

$$1/v = \frac{1}{k_5} + \frac{1}{k_9} + \frac{1}{k_{13}}$$

$$\frac{K_{UDPG}}{V} = \frac{1}{k_1}$$

$$K_{NAD_1} = \frac{k_4 + k_5}{k_3 k_5}$$

$$K_{NAD_2} = \frac{k_8 + k_9}{k_7 k_9}$$

$$K_{NAD_3} = \frac{k_{16} + k_{17}}{k_{15} k_{17}}$$

$$K_{iUDPG} = \frac{k_2}{k_1}$$

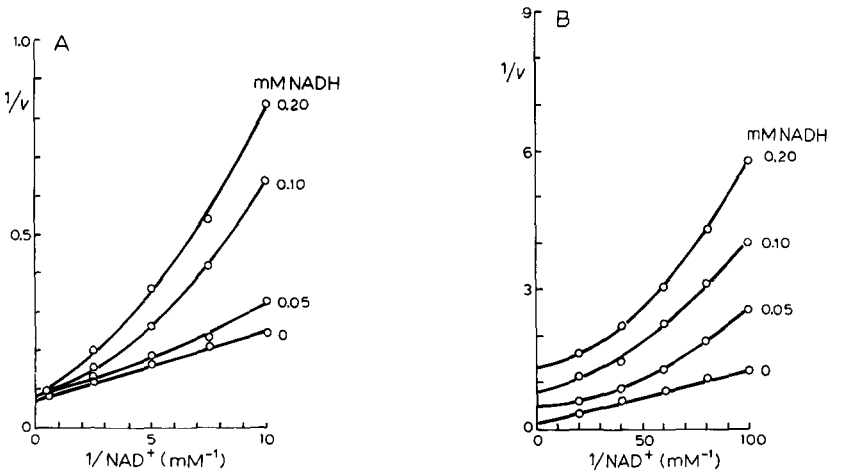


Fig. 2. Product inhibition of UDPG dehydrogenase by NADH with NAD^+ as the variable substrate and UDPG at fixed, non-saturating concentrations. The assay is described under Materials and Methods. The reciprocal of the velocity of NADH formation in μM per min is plotted as a function of the reciprocal of NAD^+ concentration in mM. The concentration of UDPG is 0.10 mM.

Figs. 3a and 3b are plots of the intercepts in Fig. 2 versus the reciprocal of UDPG concentration. When the intercepts were extrapolated from low NAD^+ concentrations, NADH gave uncompetitive inhibition of UDPG when NAD^+ was saturating. In Fig. 3b, where the intercepts were extrapolated from high

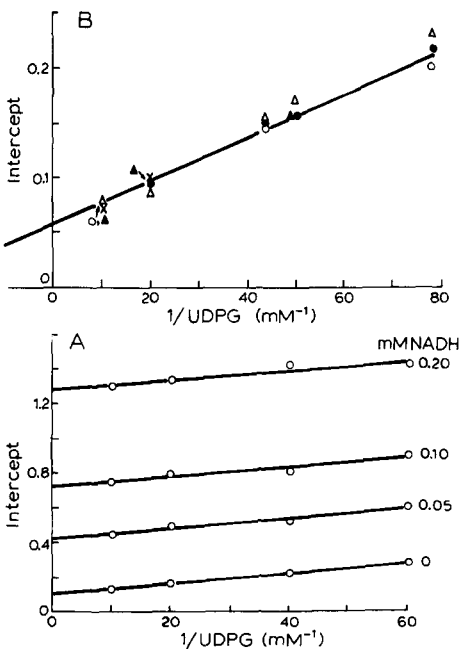


Fig. 3. Extrapolated intercepts which represent saturation with NAD^+ such as those in Fig. 2, are plotted as a function of the reciprocal of UDPG concentration at fixed levels of NADH. Points in A were extrapolated from concentrations of NAD^+ below 0.05 mM as in Fig. 2b. Points in B were extrapolated from concentrations of NAD^+ above 0.1 mM, containing the following concentrations of NADH: X, 0.20 mM; △, 0.15 mM; ●, 0.10 mM; ○, 0.025 mM; ▲, 0 mM.

NAD^+ concentrations, no inhibition of UDPG addition was observed when NAD^+ was saturating.

NADH gave non-competitive inhibition of UDPG. From least squares slope and intercept replots K_{is} = 14 and 30 μM NADH when NAD^+ concentration was held constant at 0.0167 and 0.133 mM, respectively, and K_{ii} = 80 μM NADH at both NAD^+ concentrations. The range of UDPG concentration was 0.0167—0.10 mM and of NADH 0—0.20 mM.

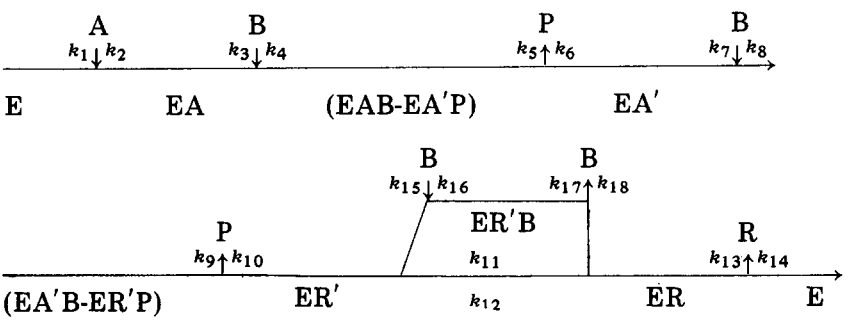
UDPGlcUA inhibition. UDPGlcUA was competitive versus UDPG at 0.25 and 0.067 mM NAD^+ with K_i = 95 μM UDPGlcUA. UDPG was varied from 0.01 to 0.10 mM and UDPGlcUA from 0 to 0.33 mM.

UDPGlcUA was non-competitive versus NAD^+. At 0.1 mM UDPG, K_{ii} = 0.55 mM and K_{is} = 0.13 mM UDPGlcUA. At 0.01 mM UDPG, K_{ii} = 0.18 mM and K_{is} = 0.06 mM UDPGlcUA. The range of NAD^+ concentration was 0.033—0.20 mM and of UDPGlcUA was 0—0.33 mM.

This is consistent with an ordered mechanism in which UDPG adds first, but not with one in which NAD^+ would add first and UDPGlcUA would act as a dead end inhibitor with the enzyme · NAD^+ complex. The latter would result in an uncompetitive inhibition pattern.

Discussion

A mechanism by which all the patterns can be explained is shown below, where A = UDPG, B = NAD^+, P = NADH, R = UDPGlcUA, A′ = UDPglucose 6-aldehyde, and ER′ is the thiol ester between the enzyme and UDPGlcUA.



The rate equation for this mechanism is given in Eqn. 1.

$$2/v = \frac{1}{k_5} + \frac{1}{k_9} + \frac{1}{k_{13}} + \frac{1}{k_1}\frac{1}{A} + \frac{k_8 + k_9}{k_7 k_9}\frac{1}{B} + \frac{k_2k_5 + k_2k_4}{k_1k_3k_5}\frac{1}{AB} + \frac{k_{14}}{k_1k_{13}}\frac{R}{A}$$

$$+ \frac{k_2k_4k_{14} + k_2k_5k_{14}}{k_1k_3k_5k_{13}}\frac{R}{AB} + \frac{k_6k_8 + k_6k_9}{k_5k_7k_9}\frac{P}{B} + \frac{k_2k_4k_6(k_8 + k_9)}{k_1k_3k_5k_7k_9}\frac{P}{AB^2}$$

$$+ \frac{k_2k_4k_6k_{14}(k_8 + k_9)}{k_1k_3k_5k_7k_9k_{13}}\frac{PR}{AB^2} + X\left[1 + \frac{k_{10}P}{k_9} + \frac{k_8k_{10}}{k_7k_9}\frac{P}{B} + \frac{k_6k_8k_{10}}{k_5k_7k_9}\frac{P^2}{B} + \frac{k_4k_6k_8k_{10}}{k_3k_5k_7k_9}\frac{P^2}{B^2}\right.$$

$$\left. + \frac{k_2k_4k_6k_8k_{10}}{k_1k_3k_5k_7k_9}\frac{P^2}{AB^2} + \frac{k_2k_4k_6k_8k_{10}k_{14}}{k_1k_3k_5k_7k_9k_{13}}\frac{P^2R}{AB^2}\right] + Y\left[\frac{k_4 + k_5}{k_3k_5}\frac{1}{B} + \frac{k_4k_6(k_8 + k_9)}{k_3k_5k_7k_9}\frac{P}{B^2}\right]$$

where $X = \dfrac{k_{16} + k_{17}}{k_{11}(k_{16} + k_{17}) + k_{15}k_{17}B}$

$$Y = \frac{k_{11}k_{17} + k_{15}k_{17}B}{k_{11}(k_{16} + k_{17}) + k_{15}k_{17}B} \tag{1}$$

All of the kinetic patterns observed are compatible with the above mechanism and equation.

A characteristic of the ordered addition of substrates is that NADH becomes an uncompetitive inhibitor versus UDPG at saturating levels of NAD^+ because the addition of NAD^+ becomes irreversible. The relevant equation which can be derived from Eqn. 1, is:

$$2/v = \frac{1}{k_5} + \frac{1}{k_9} + \frac{1}{k_{11}} + \frac{1}{k_{13}} + \frac{1}{k_1}\frac{1}{A} + \frac{k_{10}P}{k_9k_{11}} \tag{2}$$

The agreement of Fig. 3A with Eqn. 2 confirms the ordered addition with UDPG adding first.

However, at high levels of NAD^+ the extrapolated intercepts show no inhibition by NADH, as show in Fig. 3B. For all inhibition to be overcome, both steps at which NADH is released must be irreversible. The proposed mechanism accounts for this by the additional binding of NAD^+ after the release of the second NADH. This binding of NAD^+ is competitive with NADH, but otherwise does not participate in the reaction. The relevant equation to Fig. 3B is shown below.

$$2/v = \frac{1}{k_5} + \frac{1}{k_9} + \frac{1}{k_{13}} + \frac{1}{k_1}\frac{1}{A} \tag{3}$$

The overall reaction is irreversible [1,2,3]. The kinetic data firmly support the occurrence of an irreversible step after the second release of NADH, but before the release of UDPGlcUA. All of the other steps must be reversible for the mechanism to fit the data. No other mechanism could be derived which is compatible with the patterns observed. It is noteworthy that this confirms the observation that both redox steps are reversible [7]. It is postulated that this irreversible step ($k_{12} \cong k_{18} \cong 0$) is associated with the hydrolysis of a thiol ester as has been suggested for this and for other aldehyde dehydrogenases [8,10,11, 22,23].

The thiol ester hydrolysis is not only the irreversible step, but also the rate-limiting step of the reaction. From the data in Fig. 3 and Eqns. 2 and 3, it can be calculated that $k_{11} \cong 17$ μM NADH/min and that $V \cong 17$ μM NADH/min. Therefore k_{11}, the thiol ester hydrolysis, must be the rate-limiting step in the reaction sequence, equal to the observed maximum velocity. This idea is supported by several observations. The rate-limiting step occurs after the formation of UDPGlc-6-CHO [3]. The second redox reaction is readily reversible [7], indicating that it involves a thiol ester. k_{11} is similar in magnitude to the rate constants for the hydrolysis of thiol esters between papain and hippuric acid [30].

Two other 4-electron dehydrogenases, hisitidinol dehydrogenase and hydroxylmethylglutaryl-CoA reductase, have been shown to require thiol groups

[31,32]. In the case of histidinol dehydrogenase the thiol group is a part of the enzyme's structure and could well function in a fashion similar to that postulated here. For hydroxymethylglutaryl-CoA reductase the thiol is provided by CoASH, which functions exactly as in the manner postulated here for the essential thiol of UDPG dehydrogenase.

The ordered addition of the first two substrates demonstrated here for UDP-glucose dehydrogenase is well established for other dehydrogenases. In the case of horse liver alcohol dehydrogenase [24,25], pig heart malate dehydrogenase [26], beef heart and muscle lactate dehydrogenase [27], beef liver L-glutamate dehydrogenase [28], and horse liver aldehyde dehydrogenase [22], NAD^+ binds first. For yeast aldehyde dehydrogenase, the substrate rather than NAD^+ binds first [27], as is the case with the present enzyme.

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