THE MECHANISM OF ACTION OF UDPG-DEHYDROGENASE

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Received June 3, 1966

The enzyme UDPG-dehydrogenase which converts UDPG* to UDPGA, utilizing NAD^+ as the oxidizing agent, is unusual in that it appears to involve a two-step oxidation (4 electron transfer) effected by a single enzyme without the accumulation of an intermediate whose level of oxidation is between that of UDPG and UDPGA (Strominger et al., 1957). Even the purest preparations obtained by Strominger's group from calf liver (200 to 400-fold) still brought about the two-step oxidation, indicating that a single enzyme catalyzed both steps. These authors could not demonstrate the formation of an intermediate at the aldehyde oxidation level and during the course of reaction a stoichiometric relation was observed between the NADH produced and the UDPGA formed. Both observations indicate an obligatory two-step oxidation of UDPG once it becomes attached to the enzyme, i.e., the intermediate(s) remain tightly bound to the enzyme during the course of the reaction.

We would now like to present evidence that an as yet unidentified substance (XUDPG) is formed during the first part of the reaction and that this intermediate is then further oxidized to UDPGA.

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* Abbreviations: UDPG and UDPGA represent, respectively, UDP-D-glucose and UDP-D-glucuronic acid. XUDPG, XUMP and XU represent respectively structures analogous to uridine diphosphate glucose, uridine monophosphate and uracil in which the uracil moiety has been modified in an as yet undetermined fashion.

METHODS

UDPG-dehydrogenase was purified from calf liver acetone powder through step five of the procedure of Strominger et al., 1957. The preparation had a specific activity of 145 units/mg. of protein, the unitage being that of Strominger et al. Enzyme activity was determined by following the formation of NADH using a Bausch and Lomb 505 spectrophotometer. Enzyme incubations were carried out at room temperature in a 3 ml volume containing: glycine buffer, 0.3 M pH 8.7; 0.15 to 0.45 μmoles of UDPG; 1.5 to 3.0 μmoles of NAD^+ and 14.5 units of enzyme.

UDPG and NAD^+ were purchased from PL Biochemicals, Inc., Milwaukee, Wisconsin. UDPG-^{14}C was prepared from uniformly labeled glucose by the methods of Roseman et al., 1961 and Nordin et al., 1965. UDPGA-^{14}C was prepared by the action of UDPG-dehydrogenase on UDPG-^{14}C (Feingold et al., 1960). Both UDPG-^{14}C and UDPGA-^{14}C were purified by paper chromatography and UDPGA-^{14}C was also purified by paper electrophoresis.

Three solvent systems were used in the paper chromatography reported here. Solvent A, absolute ethanol, 1 M ammonium acetate, pH 7.5; 7:3 (Moffatt et al., 1961); Solvent B, absolute ethanol, methylethylketone, 0.5 M morpholinium tetraborate, pH 8.6; 7:2:3 (Carminatti et al., 1965); and Solvent C, pyridine, ethyl acetate, water; 2:5:7 upper phase (McFarren et al., 1951). Whatman #4 paper was used with solvent A and Whatman #1 with solvents B and C.

Paper electrophoresis was carried out at 600 V for 3 to 4 hours on Whatman #1 paper using two buffers. The buffers were 0.1 M bisulfite pH 4.7 (Theander, 1957) and 0.1 M sodium tetraborate pH 9.3.

RESULTS

The kinetics of formation of NADH during the course of the reaction proved unusual. If a mechanism of the type proposed by Strominger et al., 1957, is assumed, (all intermediates remaining tightly bound to the enzyme)

then the kinetics expected are those of a typical two substrate reaction (Schwert and Hakala, 1952). Such did not prove to be the case (Fig. 1, curve A). However, if the assumption is made that the reaction proceeds in two distinctly separated steps of the type:

$$UDPG + NAD^+ \rightarrow XUDPG + NADH + H^+ \quad (1)$$

$$XUDPG + NAD^+ \rightarrow UDPGA + NADH + H^+ \quad (2)$$

where reaction (2) does not take place until reaction (1) is essentially complete, and where XUDPG represents a substance at an intermediate level of oxidation, then the observed kinetics fit very well with those expected of such a mechanism (Fig. 1, curves B and C).

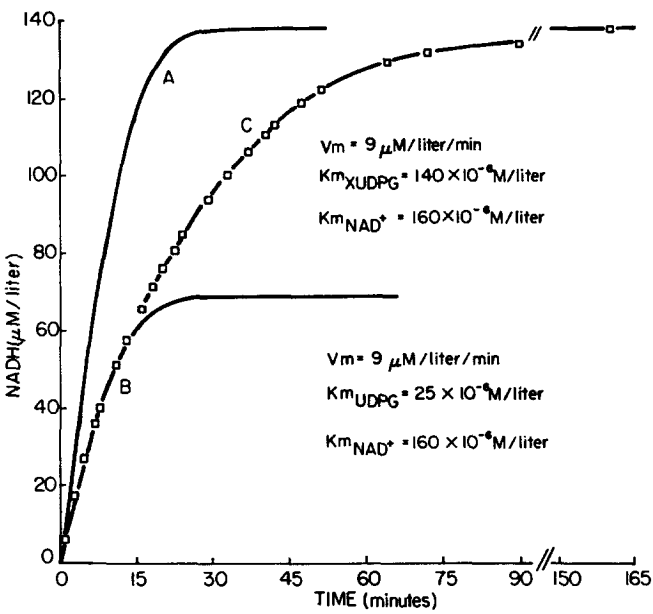

Fig. 1. Formation of NADH as a function of time during the oxidation of UDPG to UDPGA. The conditions of incubation were those described in the text. Curve A is that expected if the reaction followed typical two substrate kinetics (Schwert and Hakala, 1952); curves B and C are those expected if the reaction proceeds in two separate steps as discussed in the text. The open squares are experimentally determined points. The K_m and V_m values for curve B were determined by a Lineweaver-Burk plot (Dixon and Webb, 1958) from the first 5 minutes of the reaction. The K_m and V_m values for curve C were determined by a Henri plot (Dixon and Webb, 1958) from 20 to 90 minutes of the reaction.

The V_m for both reactions proved to be the same, as did the K_m for NAD^+; however, the K_m for UDPG was different from that of XUDPG (Fig. 1). The

failure of the enzyme to oxidize XUDPG until after UDPG has essentially disappeared can be accounted for partially on the basis of K_m values, although preliminary evidence indicates that an allosteric effect of UDPG is also very probably involved.

In view of these results an investigation of the labeled products appearing in the course of the enzyme's action on ^{14}C-labeled UDPG was undertaken. It is evident from Fig. 2 that significant amounts of UDPGA are produced only after the appearance of the first mole of NADH.

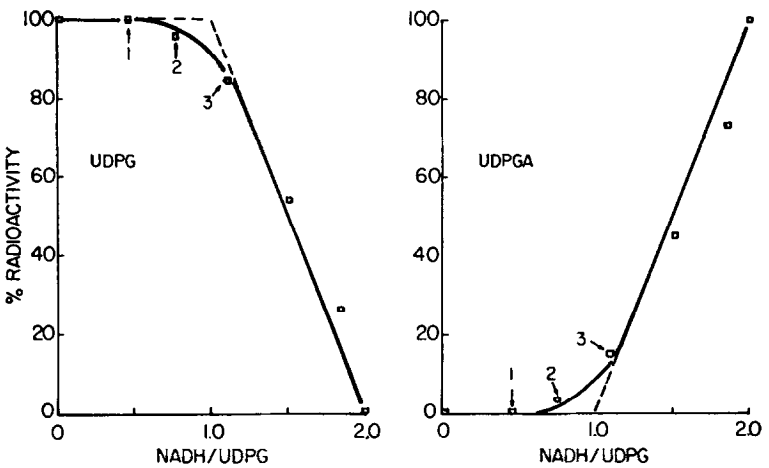

Fig. 2. Labeled products appearing during the course of the enzyme's action on ^{14}C-labeled UDPG. The products were separated by paper chromatography using solvent A and the chromatograms scanned in a Nuclear Chicago 4 π strip scanner. The ordinate is the percent of the total radioactivity on the chromatogram appearing in either the UDPG or UDPGA areas. These values were obtained by planimetric measurement of the areas under the scan curves. The abscissa is the ratio of molar concentration of NADH produced to the zero time molar concentration of UDPG. Points 1, 2 and 3 indicate the points at which the reaction mixture was sampled to carry out the further chromatographic analysis discussed in the text.

This is obviously in accord with the two-step mechanism indicated by the kinetic studies. It would appear from Fig. 2 that the amount of UDPG remains constant during the production of the first mole of NADH. This is explained very simply by the fact that solvent A does not separate UDPG from the intermediate XUDPG. In order to obtain evidence for a conversion of UDPG to XUDPG, rechromatography was carried out on the material which ran as UDPG in Fig. 2.

Specifically, the areas corresponding to UDPG on the chromatograms from samples 1, 2 and 3 (Fig. 2) were eluted and rerun in solvent B. Samples 1 and 2 were taken at times when 46.4 and 75.8% of the first mole of NADH had been produced; sample 3 at a time immediately following the production of the first mole of NADH. Chromatograms of samples 1 and 2 showed two labeled substances present. One of these had a mobility corresponding to UDPG, the other had an R_{UDPG} of 0.81 and is, presumably, XUDPG. The relative amounts of these as judged qualitatively from the areas under the scan curves are what would be expected from the proposed mechanism, namely the amounts were approximately equal in sample 1 and XUDPG predominated in sample 2. Sample 3 showed only one labeled substance and it corresponded in mobility to XUDPG, again in excellent agreement with prediction from the proposed mechanism.

A resume of our attempts to identify XUDPG is as follows: although the most probable intermediate in the oxidation of UDPG to UDPGA is UDP-6-aldehydo-glucose, it appears that the first step in the oxidation actually involves the uracil moiety of UDPG. When a solution of XUDP was hydrolyzed under conditions that produce glucose and UMP from UDPG (pH 1.0 with HCl, 10 min. at 100°), the hexose moiety present in the hydrolysate of XUDPG behaved identically with glucose upon chromatography in solvent C and upon electrophoresis in the borate and bisulfite buffer systems. A sample of 6-aldehydo-glucose, prepared by the dimethylsulfoxide oxidation of 1,2,3,4-tetraacetyl glucose (Burdon and Moffatt, 1965) clearly separated from glucose upon electrophoresis in bisulfite buffer.

When the same hydrolysate was chromatographed in solvent A, two UV absorbing spots appeared, one with the same mobility as UMP, the other with the mobility of uracil. A control sample of UDPG submitted to the same hydrolysis procedure gave only one UV absorbing material, UMP. The apparent ease of hydrolysis of XUDPG suggests that it is the uracil moiety that is altered in the first reaction. Spectrophotometric examination of eluates of the two UV absorbing spots from the XUDPG hydrolysis mixture showed they

were not identical with UMP and uracil (Fig. 3).

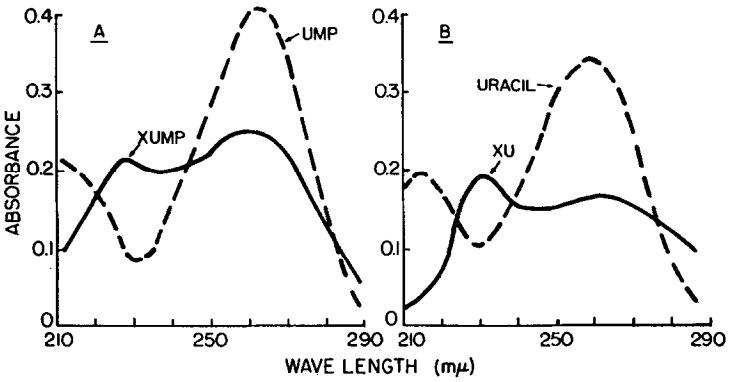

Fig. 3A. Absorbtion spectra of UMP and XUMP.
Fig. 3B. Absorbtion spectra of uracil and XU.

It is felt that these two UV absorbing materials are probably XUMP and XU. This possibility is increased by the observation that the material eluted from the XUMP spot was completely converted to XU upon further hydrolysis (pH 1, 100° for 45 min.).

It would appear, upon the basis of the evidence reported here, that the enzyme UDPG-dehydrogenase acts upon its substrate in two distinct and clearly separated steps. In the first of these the uracil moiety of UDPG is oxidized at the expense of NAD^+ to produce an as yet uncharacterized substance (XUDPG) which appears to differ from UDPG only in that the uracil moiety has been altered. In the second step, which occurs only when the first is essentially complete, XUDPG is oxidized with another mole of NAD^+ and in the course of this reaction the oxidized uracil moiety is restored to its original condition while the 6-carbon of the glucose moiety is oxidized to a carboxyl group. The final product of the reaction is thus UDPGA.

Acknowledgments. This investigation was supported by Public Health Service Research Grant GM 09293, from the National Institute of General Medical Sciences.

Scientific Journal Series No. 5980, Agricultural Experiment Station, University of Minnesota, St. Paul, Minnesota.

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