Activation of liver alcohol dehydrogenase by glycosylation

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(Received 18 May 1982/Accepted 8 October 1982)

D-Fructose and D-glucose activate alcohol dehydrogenase from horse liver to oxidize ethanol. One mol of D-[U-¹⁴C]fructose or D-[U-¹⁴C]glucose is covalently incorporated per mol of the maximally activated enzyme. Amino acid and N-terminal analyses of the ¹⁴C-labelled glycopeptide isolated from a proteolytic digest of the [¹⁴C]glycosylated enzyme implicate lysine-315 as the site of the glycosylation. ¹³C-n.m.r.-spectroscopic studies indicate that D-[¹³C]glucose is covalently linked in N-glucosidic and Amadori-rearranged structures in the [¹³C]glucosylated alcohol dehydrogenase. Experimental results are consistent with the formation of the N-glycosylic linkage between glucose and lysine-315 of liver alcohol dehydrogenase in the initial step that results in an enhanced catalytic efficiency to oxidize ethanol.

Chemical (non-enzymic) glycosylation of proteins has attracted considerable research activities recently because of its physiological implications in correlating glycosylated haemoglobin (Bunn *et al.*, 1978) and lens crystallins (Stevens *et al.*, 1978) with diabetes mellitus. In view of reports that fructose stimulates hepatic ethanol metabolism (Brown & Forrest, 1972; Damgaard *et al.*, 1973; Scholz & Nohl, 1976), we have investigated the effect of glycoses on liver alcohol dehydrogenase (EC 1.1.1.1), which is the major enzyme catalysing the initial step of hepatic ethanol oxidation. Unlike related studies on the chemical glycosylation of non-enzymic proteins, which are difficult to monitor, the enzymic proteins offer the advantages that changes in enzymic activities can be correlated directly with the process of glycosylation. We now report the activation of liver alcohol dehydrogenase by D-fructose and D-glucose. Evidence for the formation of glucosylated enzyme with an enhanced oxidative activity is presented.

Materials and methods

Chemicals

Alcohol dehydrogenase from horse liver (once crystallized), papain, aminopeptidase, prolidase, NAD⁺, D-fructose, 2-deoxy-D-glucose and *p*-nitrophenyl- α - and β -D-glucopyranosides were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). D-Glucose was the product of Fisher Scientific Co. (Ottawa, Ont., Canada). Glucose 1-phosphate and glucose 6-phosphate were purchased from ICN Nutritional Biochemicals (Cleveland, OH, U.S.A.). Ethanol (100%) came from Consolidated Alcohol

(Toronto, Ont., Canada). D-[U-¹⁴C]Fructose and D-[U-¹⁴C]glucose were provided by New England Nuclear (Boston, MA, U.S.A.). D-[1-¹³C]Glucose and ²H₂O were supplied by Merck, Sharp and Dohme (Dorval, Que., Canada). Bio-Gel P-6 was the product of Bio-Rad Laboratories (Richmond, CA, U.S.A.). Ninhydrin reagent was prepared by mixing 200 ml of acetone containing 200 mg of ninhydrin and 30 ml of acetic acid/water (1:2, v/v) containing 200 mg of cadmium acetate.

Glycosylation

A 15 mg portion of alcohol dehydrogenase in 1.5 ml of 0.20 M-potassium phosphate buffer, pH 8.0, was incubated at 25°C with an appropriate amount (usually 9–10 mg) of D-fructose or D-glucose. Samples (0.10 ml) were withdrawn at time intervals and filtered through a Bio-Gel P-6 column (14 cm $\times$ 1 cm) equilibrated with 0.10 M-potassium phosphate buffer, pH 7.0. The filtrates were assayed for the dehydrogenase activity (Tsai *et al.*, 1974). The controls without D-glycoses were prepared in parallel experiments. The incorporation of D-[U-¹⁴C]fructose or D-[U-¹⁴C]glucose was followed by incubating 10 mg of D-fructose or D-glucose containing 12.5 μ Ci (2.2 mCi/mmol) of D-[U-¹⁴C]fructose or D-[U-¹⁴C]glucose per 15 mg of enzyme. At time intervals, samples (100 μ l) were delivered on to Whatman 3MM discs (2.3 cm diam.), which were washed successively with 10% (w/v) trichloroacetic acid three times followed by once each with ethanol, ethanol/diethyl ether (1:1, v/v) and diethyl ether. The air-dried discs were placed into counting vials containing 10 ml of Aquasol, and radioactivity was measured with a Packard scintillation counter.

Identification of glucosylated peptides

To isolate the glucosylated peptide fragment, D-[U-¹⁴C]glucose-treated alcohol dehydrogenase was hydrolysed first with papain and then with a mixture of leucine aminopeptidase and prolidase (Hill & Schmidt, 1962). The ¹⁴C-labelled glycopeptide was isolated from the proteolytic digest by two cycles of paper chromatography with butanol/acetic acid/water (40:7:15, by vol.) as the development solvent and paper electrophoresis with pyridine/acetic acid/water (1:10:289, by vol.), pH 3.6, or pyridine/acetic acid/water (25:1:225, by vol.), pH 6.5, as buffer in a Savant flat-plate electrophoresis apparatus. Peptides in a parallel run were located with a ninhydrin reagent, and the radioactive area was monitored with a Packard model 7201 radiochromatogram.

The ¹⁴C-labelled glycopeptide was hydrolysed with 6.0M-HCl at 110°C for 20h for amino acid analysis with a Beckman 119 BL amino acid analyser or subjected to N-terminal analysis (Silver, 1977).

¹³C-n.m.r.-spectroscopic studies

[¹³C]Glucosylated alcohol dehydrogenase was prepared by incubating 25mg of the enzyme with 15mg of D-[1-¹³C]glucose in 2.0ml of 0.10M-

potassium phosphate buffer, pH 8.0, for 2h. The incubation mixture was dialysed, concentrated and adjusted to the original volume with ²H₂O. Ethanol (100%) or acetone (1 equivalent in ¹³C) was added as an internal carbon standard. ¹³C-n.m.r. spectra (35 × 10³–40 × 10³ scans) were recorded with a Varian XL100-12 n.m.r. spectrometer equipped with Nicolet Fourier-transform and TT-100 Data systems. The chemical shifts of the internal standards were calibrated with the external standard tetramethylsilane.

Enzymic assay and kinetic studies

The dehydrogenase activity was assayed by following the rate of NADH formation of a reaction mixture containing 1.0mM-NAD⁺ and 10mM-ethanol in 0.10M-sodium pyrophosphate buffer, pH 9.0, at 25°C, by using a Beckman model 25 spectrophotometer.

Kinetic studies were conducted as described previously (Tsai, 1978). Initial velocities were analysed according to:

$$v = \frac{V[A][B]}{[A][B] + K_a[B] + K_b[A] + K_{ia}K_b}$$

where V , K_a , K_b and K_{ia} are maximum velocity, Michaelis constant for A (NAD⁺), Michaelis con-

Fig. 1. Activation of liver alcohol dehydrogenase by D-fructose

Liver alcohol dehydrogenase (15 mg) was incubated with a 325 molar excess of D-fructose (O) or D-[U-¹⁴C]fructose (●) at pH 8.0 and 25°C. At time intervals, samples were withdrawn for measurements of dehydrogenase activity and ¹⁴C incorporation. The inset shows the dose-response relationship of activation (initial 10 min) plotted versus molar ratio of D-fructose to alcohol dehydrogenase.

stant for B (ethanol) and inhibition constant for A respectively. Enzyme concentrations were determined spectrophotometrically by using $\epsilon_{280} = 3.53 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ with a molecular weight of 84 000 (Dalziel, 1958).

Results

An incubation of alcohol dehydrogenase with D-fructose at pH 8.0 results in an enhanced dehydrogenase activity (Fig. 1). The activation, which reached a maximum within 10 min, was accompanied by a rapid incorporation of approx. 1 mol of D-[U- ^{14}C]fructose per mol of enzyme. Subsequent gradual incorporation led to a slight decrease in the activity. The inset in Fig. 1 shows the dose-response curve of the fructose effect. The time-dependent stimulations of alcohol dehydrogenase activity, in the oxidation of ethanol by D-glucose, follow a similar profile except that the same degree of activation was achieved in 40–45 min. Of the two phosphate derivatives, glucose 6-phosphate activates alcohol dehydrogenase whereas glucose 1-phosphate is ineffective. A stimulatory effect was also observed for 2-deoxy-D-glucose.

To examine whether an identical amino acid residue was affected by D-fructose as well as D-glucose in the activation, double-modification experiments were performed. Liver alcohol dehydrogenase that had initially been activated by D-glucose was subsequently treated with D-[U- ^{14}C]fructose. The failure of D-fructose to stimulate the D-glucose-treated enzyme suggests that the amino acid residue responsible for the activation had been glucosylated and was not available for fructosylation. An identical effect was observed for the reverse sequence. Sodium cyanoborohydride, which had been used to prepare synthetic glycoprotein (Schwartz & Gray, 1977), did not deter the stimulatory effect of D-glucose.

The incorporation of D-[U- ^{14}C]fructose and D-[U- ^{14}C]glucose (result not shown) into the trichloroacetic acid-insoluble protein and the non-diffusible fraction implicates the formation of a covalent linkage between alcohol dehydrogenase and D-glycoses during the activation. Fig. 2 shows that D-[U- ^{14}C]glucose remained attached to one major peptide and two or three minor peptides. An identical result was obtained for the D-[U- ^{14}C]fructosylated enzyme. The major ^{14}C -labelled glycopeptide was isolated for amino acid and N-terminal analyses (Table 1). Although the major ^{14}C -labelled glycopeptides, peptides I and II, isolated from different experiments differ in their chain length, results from the amino acid analyses suggest that they were probably derived from an identical fragment.

To elucidate the structure of the covalently linked

Fig. 2. Radioactive ^{14}C -labelled glycopeptides from the proteolytic digest of D-[U- ^{14}C]glucose-activated alcohol dehydrogenase

Liver alcohol dehydrogenase was incubated with D-[U- ^{14}C]glucose to reach the maximum enzymic activity. [^{14}C]Glucosylated alcohol dehydrogenase was isolated and hydrolysed with a mixture of proteolytic enzymes. The digest was subjected to paper chromatography (a). Most of the radioactivity was associated with peptides (P). Protein remained on the origin (O), and A represented amino acid regions. No radioactivity was detected between P and A, where D-glucose was localized. The peptides (P) were subjected to electrophoresis with either one of the buffer systems: (b) pyridine/acetic acid/water (25:1:225, by vol.), pH 6.5, at 1.5 kV for 2 h, or (c) pyridine/acetic acid/water (1:10:289, by vol.), pH 3.6, at 2.0 kV for 2 h. The major radioactive band was isolated for subsequent studies indicated in Table 1.

D-glucose by ^{13}C -n.m.r. spectroscopy, alcohol dehydrogenase was glycosylated with D-[1- ^{13}C]glucose. Fig. 3 shows the proton-decoupled ^{13}C -n.m.r. spectrum of the purified [1- ^{13}C]glucosylated enzyme. Two ^{13}C -n.m.r. resonance peaks are detectable at 63.3 and 73.0 p.p.m., confirming the formation of a covalent linkage. Their chemical shifts are clearly different from ^{13}C -1 resonance peaks of free α - and β -glucoses (Dorman & Robert, 1970) but within the range of N-glucosides (Dill & Allerhand, 1979) and an Amadori product (Funcke & Klemer, 1975). The N-glucosidic and Amadori-rearranged structures can be readily differentiated by the proton-coupled

Table 1. Analyses of ^{14}C -labelled glycopeptides from the proteolytic digests of [^{14}C]glucosylated liver alcohol dehydrogenase

The [^{14}C]glucosylated alcohol dehydrogenase (100 mg in 20 ml) was denatured by heating at 90°C for 3 min, followed by maintenance at 40°C for 10 min. The suspension of denatured enzyme was incubated with papain (1.0%) in 10 mM-CN $^-$ at pH 7.0 for 24–36 h, at which time the mixture was completely clarified. The pH was adjusted to 8.4–8.6, and 0.25 vol. of 25 mM-Tris buffer containing 25 mM-MgCl $_2$ was added. The proteolysis was continued by the addition of leucine aminopeptidase (700 units) and prolidase (200 units) at 40°C for 24 h. The digest was clarified by centrifugation and freeze-dried. The ^{14}C -labelled glycopeptide was isolated by the combination of high-voltage paper electrophoresis and descending paper chromatography as described in the text. ^{14}C -labelled glycopeptides of different chain lengths, peptide I and peptide II, which are the major radioactive peptides in two experiments respectively, were analysed for amino acid composition after acid hydrolysis, under which conditions tryptophan was destroyed. The peptide II from the second experiment was also subjected to the *N*-terminal analysis.

	Amino acid	Molar ratio
Composition, peptide I	Alanine	1
	Arginine	1
	Glycine	2
	Serine	1
	Threonine	1
Composition, peptide II	Glycine	1
	Threonine	1
<i>N</i> -Terminal, peptide II	Threonine

Fig. 3. Proton-decoupled ^{13}C -n.m.r. spectrum of [$1\text{-}^{13}\text{C}$]glucosylated liver alcohol dehydrogenase

Signals at 17.6 and 58.3 p.p.m. correspond to ethanol used as an internal standard, which was used to correct for chemical shifts against the external standard, tetramethylsilane. Peaks at 63.3 and 73.0 p.p.m. arise from C-1 of covalently linked glucose. The spectrum was acquired after 39005 scans.

Fig. 4. Off-resonance ^{13}C -n.m.r. spectrum of [$1\text{-}^{13}\text{C}$]glucosylated liver alcohol dehydrogenase

Chemical shifts were corrected against the external standard, tetramethylsilane. Acetone was used as the internal standard, which gave the doublet at 32.3 p.p.m. The proton-coupled multiplicities of the covalently linked [$1\text{-}^{13}\text{C}$]glucosyl resonances are shown at 63.3 (triplet) and 73.0 (doublet) p.p.m.

splittings due to a difference in the number of hydrogen atoms attached to the C-1 atom. The off-resonance ^{13}C -n.m.r. spectrum is shown in Fig. 4. The doublet at 73.3 p.p.m. characterizes the *N*-glucosidic C-1, and the triplet at 63.3 p.p.m. corresponds to the C-1 of the Amadori-rearranged product.

The glycosylated enzymes were isolated for kinetic characterization. Although the native alcohol dehydrogenase was able to hydrolyse *p*-nitrophenyl α - and β -D-glucopyranosides slowly ($0.59 \times 10^{-3} \pm 0.09 \times 10^{-3} \text{ min}^{-1}$) at pH 8.0, this glucosidase activity of the enzyme was completely abolished by glycosylation. The glycosylation appears to increase V , K_m and K_{ia} slightly, although the effect is comparable in magnitude with the deviation of the determinations.

Discussion

In the present study we have demonstrated the stimulation of alcohol dehydrogenase activity, in the oxidation of ethanol, by D-fructose and D-glucose via the formation of a covalent linkage. Although the concentration required and the magnitude of activation detract from the importance of the glycosylation of alcohol dehydrogenase as a viable explanation for the physiological fructose effect, the glycosylation provides an interesting possibility for investigators to affect enzymic activities post-translationally (Wold, 1981).

The formation of a covalent linkage between alcohol dehydrogenase and D-glucose (or D-fruc-

tose) is substantiated by the isolation of the ^{14}C -labelled glycopeptide from the D-[U- ^{14}C]glucosylated (or D-[U- ^{14}C]fructosylated) enzyme. The site of glycosylation is deduced from the results of amino acid and *N*-terminal analyses of the purified ^{14}C -labelled glycopeptide in comparison with the known sequence of alcohol dehydrogenase (Jörvall, 1970). Since tryptophan and glucosylated lysine are destroyed during hydrolysis, the structure for the ^{14}C -labelled glycopeptide II is most probably:

The glucosylation of the lysine residue is also consistent with ^{13}C -n.m.r. results. Lys-315 is located in the cleft of the subunit-interaction region. It is adjacent to Trp-314, which interacts with Trp-314 from the other subunit (Eklund *et al.*, 1976). The close proximity of the two Lys-315 residues may explain the half-of-sites reactivity and consequently the specificity of the glycosylation.

^{13}C -n.m.r.-spectroscopic studies of the D-[1- ^{13}C]glucosylated alcohol dehydrogenase provide the compelling evidence for glycosylation and structures of the glucosylated enzyme. The absence of ^{13}C -1-n.m.r. resonance signal in the 90–100 p.p.m. region (Dorman & Robert, 1970) indicates that D-[1- ^{13}C]glucose is covalently linked. The proton-decoupled ^{13}C -n.m.r. spectrum shows two ^{13}C -n.m.r. resonance signals in a ratio of 3:1 with chemical shifts of 63.3 and 73.0 p.p.m. respectively, representing *N*-glucoside and/or Amadori product. The *N*-glucosidic C-1 is a methine carbon atom that gives a doublet by coupling to the proton, whereas

the Amadori-rearranged C-1 is a methylene carbon atom that couples to two protons, giving a triplet. The off-resonance ^{13}C -n.m.r. experiment suggests that the resonance at 63.3 p.p.m. arises from the Amadori rearrangement and that the resonance at 73.0 p.p.m. results from the *N*-glucoside. It is worthy of note that only one anomeric *N*-glucoside was detected from the ^{13}C -n.m.r. spectrum.

The glycosylation of non-enzymic proteins has been demonstrated to occur at the *N*-terminal amino acid (Koenig *et al.*, 1977) or ϵ -amino group of lysine (Day *et al.*, 1979). In all these cases, the Amadori product was detected and reaction sequences leading to its formation had been stipulated (Koenig *et al.*, 1977; Day *et al.*, 1979). The present work with the enzymic protein reveals two glucosylated structures. An easy monitoring of changes in alcohol dehydrogenase activities enables us to correlate the stimulatory effect with the structure and process of glycosylation. The following results are consistent with the formation of the *N*-glucosidic alcohol dehydrogenase in the initial step of glycosylation, which is responsible for the activation. (1) Glucose 6-phosphate stimulates alcohol dehydrogenase activity, whereas glucose 1-phosphate, with C-1 blocked, is ineffective. (2) 2-Deoxy-D-glucose, which does not undergo Amadori rearrangement, being without the C-2-hydroxy group, nevertheless stimulates alcohol dehydrogenase activity. (3) Sodium cyanoborohydride, which reduces the imine intermediate (Borch *et al.*, 1971) and therefore blocks the Amadori rearrangement, does not deter the activation. (4) The glycosylation abolishes the glucosidase activity of alcohol dehydrogenase, which hydrolyses the *p*-nitrophenyl 1-glucosidic linkage.

Scheme 1. Proposed mechanism for glycosylation of liver alcohol dehydrogenase
Abbreviation: ADH, alcohol dehydrogenase.

The specificity of glycosylation is manifested by the unusual nucleophilicity of Lys-315, presumably aided by the adjacent Arg-312 of the opposite subunit. The initial reaction between the ϵ -amino group of Lys-315 and C-1 of glucose yields the *N*-glycosidic alcohol dehydrogenase that accounts for the initial phase of activation (Scheme 1). It is followed by the slow Amadori rearrangement. The *N*-glycosidation stimulates alcohol dehydrogenase activity in the oxidation by increasing catalytic efficiency while decreasing its affinity for the nicotinamide coenzyme.

This work was supported by a grant from the Natural Sciences and Engineering Research Council (N.S.E.R.C.) of Canada. J. H. W. is the holder of an N.S.E.R.C. graduate scholarship.

References

- Borch, R. F., Betstein, M. D. & Durst, H. D. (1971) *J. Am. Chem. Soc.* **93**, 2987–2904
- Brown, S. S. & Forrest, J. A. H. (1972) *Lancet* **ii**, 898–899
- Bunn, H. F., Gabbay, K. H. & Gallop, P. M. (1978) *Science* **250**, 21–27
- Dalziel, K. (1958) *Acta Chem. Scand.* **12**, 459–464
- Damgaard, S. E., Lundquist, F., Tonnesen, K., Hansen, F. V. & Strestoft, L. (1973) *Eur. J. Biochem.* **33**, 87–97
- Day, J. F., Thorpe, S. R. & Baynes, J. W. (1979) *J. Biol. Chem.* **254**, 595–597
- Dill, K. & Allerhand, A. (1979) *FEBS Lett.* **107**, 26–29
- Dorman, D. E. & Robert, J. D. (1970) *J. Am. Chem. Soc.* **92**, 1355–1361
- Eklund, H., Nordstrom, B., Zepperzauer, E., Soderlund, G., Ohlsson, I., Boiwe, T., Soderberg, B.-O., Tapia, O. & Branden, C.-I. (1976) *J. Mol. Biol.* **102**, 27–59
- Funcke, W. & Klemer, A. (1975) *Justus Liebig's Ann. Chem.* 1323–1235
- Hill, R. L. & Schmidt, W. R. (1962) *J. Biol. Chem.* **237**, 389–396
- Jörvall, H. (1970) *Eur. J. Biochem.* **16**, 25–40
- Koenig, R. J., Blabstein, S. H. & Cerami, A. (1977) *J. Biol. Chem.* **252**, 2992–2997
- Scholz, R. & Nohl, H. (1976) *Eur. J. Biochem.* **63**, 449–458
- Schwartz, B. A. & Gray, G. R. (1977) *Arch. Biochem. Biophys.* **181**, 542–549
- Silver, J. (1977) *Methods Enzymol.* **47**, 247–260
- Stevens, V. J., Rouzer, C. A., Monnier, V. M. & Cerami, A. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2918–2922
- Tsai, C. S. (1978) *Biochem. J.* **173**, 483–496
- Tsai, C. S., Tsai, Y. H., Lauzon, G. & Cheng, S. T. (1974) *Biochemistry* **13**, 440–443
- Wold, F. (1981) *Annu. Rev. Biochem.* **50**, 783–814