SHORT COMMUNICATIONS

BBA 63321

Kinetic isotope effects in enzymatic oxidations of D-[1-H]glucose and D-[1-^{2}H]glucose

Many enzyme systems have been isolated from animals, plants and microorganisms, which convert glucose and other aldoses to the corresponding lactones. The mechanism of this oxidation has received considerable attention, particularly in the case of the fungal glucose oxidase (β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4). Cleavage of the C-1–H bond appeared to be a rate-determining step since preliminary studies indicated a marked kinetic isotope effect in the over-all reaction when D-[1-^{2}H]glucose and D-[1-^{3}H]glucose were used as substrates for fungal glucose oxidases[1–3].

It was of interest to extend such studies to other systems, especially in view of the wide variation in coenzyme requirements. For the fungal system, the coenzyme is FAD. For liver glucose dehydrogenase (β-D-glucose:NAD(P) oxidoreductase, EC 1.1.1.47) both NAD and NADP function as coenzymes, the former being usually more effective[4]. In bacteria, FAD has been implicated as coenzyme for a soluble lactose dehydrogenase obtained from subcellular particles of *Pseudomonas graveolens*[5]. NADP-dependent, soluble glucose dehydrogenases have been reported in *Acetobacter suboxydans*[6,7], *Pseudomonas fluorescens*[7], and *Pediococcus pentosaceus*[8] grown under aerobic conditions. DOUDOROFF and his colleagues[9–11] have found soluble D-galactose, D-arabinose, and L-arabinose dehydrogenases in *P. saccharophila* which were all NAD linked; other workers[12] have found NADP-linked dehydrogenases in strains of *P. saccharophila* derived from that of DOUDOROFF. Furthermore, in *Bacterium anitratum*, it has been shown that FAD, FMN, NAD, NADP and heme-type proteins do not function as active prosthetic groups in highly purified preparations of the soluble glucose dehydrogenase[13]. The coenzyme in this case may be a novel substituted naphthoquinone.

Materials used in this work were as follows. D-[1-^{2}H]Glucose was prepared by sodium amalgam reduction of D-glucono-δ-lactone using a dioxane–^2H_2O mixture. The α anomeric form was crystallized from ethanol, the β form from pyridine by conventional methods. The anomeric purity of these materials was shown to be $> 98\%$ by gas chromatography[14]. Fungal glucose oxidase with $Q_{O_2} = 79,550$ μl/h per mg enzyme at 25°, was obtained from C. F. Boehringer and Söhne, Mannheim. This material is about 43% pure when compared to the crystallized enzyme from *Aspergillus niger*[15]. Beef liver glucose dehydrogenase was partially purified by the method of VAN BUREN[16]. The absorbance change at 340 mμ was 0.866/min per mg protein using 500 μmoles equilibrium glucose, 0.5 μmole NAD^+, and 1.0 ml pH 8.0, 0.2 M Tris–maleate buffer in a total volume of 3.0 ml at 35°. A solubilized dehydrogenase was prepared from particles of *Pseudomonas quercito-pyrogallica*[17] by treatment with deoxycholic acid; a partial purification was carried out essentially by the technique

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

TABLE I

KINETIC ISOTOPE EFFECT FOR GLUCOSE OXIDASE AND VARIOUS GLUCOSE DEHYDROGENASES

Enzyme	*Amount of enzyme (mg)*	*Glucose substrate*	*Substrate concn. (M)*	*NAD^+ or DCIP concn. (M)*	*Solvent*	*pH or p^2H*	*Temperature*	*Kinetic isotope effect ($k_H/k_{^2H}$)*
Fungal glucose oxidase*	0.1	β	$5.55 \cdot 10^{-3}$		H_2O	5.6	25°	2.5
	0.1	β	$5.55 \cdot 10^{-3}$		H_2O	5.6	25°	2.2
	0.1	β	$5.55 \cdot 10^{-3}$		^2H_2O	5.6**	25°	3.1
	0.1	α	$5.55 \cdot 10^{-3}$		H_2O	5.6	25°	1.7
	0.02	Equil.	$6.66 \cdot 10^{-2}$		H_2O	5.6	25°	2.9
Beef liver glucose dehydrogenase	1.0	Equil.	$1.66 \cdot 10^{-1}$	$1.66 \cdot 10^{-4}$†	H_2O	8.0***	35°	2.7
	1.0	Equil.	$1.66 \cdot 10^{-1}$	$1.66 \cdot 10^{-4}$†	^2H_2O	7.6	35°	1.7
	0.1	Equil.	$1.66 \cdot 10^{-1}$	$1.66 \cdot 10^{-4}$†	H_2O	8.0	35°	3.2
	0.1	Equil.	$1.66 \cdot 10^{-1}$	$1.66 \cdot 10^{-4}$†	^2H_2O	7.6	35°	3.4
B. anitratum glucose dehydrogenase	0.002	β	$3.33 \cdot 10^{-3}$	$4.0 \cdot 10^{-5}$††	H_2O	6.0	25°	1.6
	0.002	α	$3.33 \cdot 10^{-3}$	$4.0 \cdot 10^{-5}$††	H_2O	6.0	25°	1.3
	0.002	Equil.	$3.33 \cdot 10^{-3}$	$4.0 \cdot 10^{-4}$††	H_2O	6.0	25°	3.3
	0.002	α	$3.33 \cdot 10^{-3}$	$4.0 \cdot 10^{-4}$††	H_2O	6.0	25°	1.7
P. quercito-pyrogallica glucose dehydrogenase	0.3	Equil.	$3.33 \cdot 10^{-3}$	$3.0 \cdot 10^{-4}$††	H_2O	5.6	35°	1.1
	0.3	Equil.	$3.33 \cdot 10^{-3}$	$3.0 \cdot 10^{-4}$††	^2H_2O	6.0	35°	1.1

* This enzyme was assayed manometrically by following the consumption of oxygen. All of the other enzyme assays were carried out spectrophotometrically.

** The p^2H optimum of the fungal glucose oxidase was determined to be 5.6.

*** The pH optimum of this preparation was determined to be 8.0–8.4, while the p^2H optimum was 7.6–7.7.

† NAD^+.

†† DCIP.

of NISHIZUKA AND HAYAISHI[5]. The material had an activity of 0.4 μmole 2,6-dichlorophenolindophenol (DCIP) reduced/min per mg protein when tested with 160 μmoles glucose, 0.3 μmole DCIP and 1.5 ml phosphate buffer (pH 5.6), final concentration 0.05 M at 35°. Highly purified samples of *B. anitratum* glucose dehydrogenase, prepared as previously described[13], had activity of 430 μmoles DCIP reduced/min per mg protein when tested with 10 μmoles glucose, 0.12 μmole DCIP and 3 ml pH 6.0 phosphate buffer, final concentration 0.05 M at 25°.

The results, summarized in Table I, indicate that the fungal glucose oxidase and the glucose dehydrogenases from beef liver and *B. anitratum*, show a marked kinetic isotope effect for the over-all reaction. The effect varies somewhat with the experimental conditions; the range of values is from 1.3 to 3.4. Low values are observed in runs using the α anomer of D-glucose. Since this is not the preferred substrate of the enzyme, it is possible that the kinetic isotope effect is masked by the limited amount of β anomer available through mutarotation. A similar range of values was found in the small number of experiments carried out in solvent ^2H_2O (1.7–3.4). In contrast to these results, no real evidence of an isotope effect was obtained with the solubilized dehydrogenase obtained from *P. quercito-pyrogallica* particulates. This preparation was not as highly purified as the others used in this study, so this result must be regarded as tentative.

After the completion of this work, a detailed kinetic study of the oxidation of

D-[1-^{2}H]glucose by pure fungal glucose oxidase was published by BRIGHT AND GIBSON[18]. They concluded that the precise site of the kinetic isotope effect is on k_2 in the flavin reduction step shown below (G^2H = β-D-[1-^{2}H]glucose).

$$E_{ox} + G^2H \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{ox}\text{-}G^2H \xrightarrow{k_2} E_{red}\text{-}P$$

The actual value of the effect for this half-reaction is between 10 and 15 at 3°. The maximum apparent effect for the over-all reaction is much reduced because with D-[1-H]glucose the reaction behaves essentially as if no E_{ox}–GH were formed. Two special cases of the general steady-state rate equation are therefore required for D-[1-H]glucose and D-[1-^{2}H]glucose. A further consequence is that the over-all isotope effect is very dependent on concentrations of glucose and oxygen. Under conditions comparable to those used by us[2], BRIGHT AND GIBSON calculate a value of 3.4 for the over-all isotope effect, which agrees well with the upper values we have observed. Since the value of the over-all effect is the same for the fungal glucose oxidase, the liver glucose dehydrogenase, and the *B. anitratum* glucose dehydrogenase, it appears likely that all of these enzymes will be generally similar in reaction mechanism.

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Received February 26th, 1968