Investigation of α -deuterium kinetic isotope effects on the purine nucleoside phosphorylase reaction by the equilibrium-perturbation technique

Pertti K. LEHIKONEN,* Michael L. SINNOTT*† and Thomas A. KRENITSKY†

*Department of Organic Chemistry, University of Bristol, Cantocks Close, Bristol BS8 1TS, U.K.,

and †The Wellcome Research Laboratories, Burroughs Wellcome Co., 3030 Cornwallis Road, Research Triangle Park, NC 27709, U.S.A.

1. α -Deuterium kinetic isotope effects on the phosphorolysis of inosine catalysed by *Escherichia coli* purine nucleoside phosphorylase were measured by the equilibrium-perturbation technique, by using the change in absorbance at 250 nm (approx. 20%). 2. Values of $^2\text{H}(V/K)$ of 1.13₉ at pH 5.0, 1.10₅ at pH 6.1, 1.09₄ at pH 7.3, 1.08 at pH 8.4 and 1.16₄ at pH 9.4 were obtained. 3. These are compared with literature α -deuterium kinetic isotope effects for this and related reactions. 4. The equilibrium constant, defined as $[\text{inosine}] \cdot [\text{H}_2\text{PO}_4^-] / [\text{hypoxanthine}][\alpha\text{-RibfOPO}_3\text{H}^-]$, is 46 at 25 °C. 5. *N*-3- β -D-Ribofuranosylhypoxanthine, an impurity in chemically synthesized inosine, is a substrate.

INTRODUCTION

α -Deuterium kinetic isotope effects (α DKIEs) are a widely used probe of enzymic and non-enzymic reaction mechanisms (see, e.g., Melander & Saunders, 1980; Cleland, 1982a). They have their origin in a weakening of a C–H bending vibration as the hybridization changes from sp^3 to sp^2 . The degree to which the transition state of a glycosyl transfer resembles the glycosyl cation can be inferred from the α DKIE, but results obtained by Knier & Jencks (1980) and Craze *et al.* (1978) show that inferences should be drawn from the precise values of α DKIEs only with great caution. These authors found that the non-enzymic nucleophilic displacements of a leaving group X from $\text{CH}_3\text{OCH}_2\text{--X}$ were bimolecular reactions in which the incoming nucleophile participated very little and in which the methoxymethyl moiety at the transition state strongly resembled the methoxymethyl cation. Nonetheless, α DKIEs varied with the chemical nature of the ‘preassociated’ nucleophile, ‘soft’ nucleophiles giving rise to larger α DKIEs (Knier & Jencks, 1980). Thus for $\text{X} = ^+\text{NMe}_2\text{mC}_6\text{H}_4\text{NO}_2$ α DKIEs (per ^2H atom) were 1.07 for acetate and hydroxide, 1.08 for phenoxide and 1.11 for water; for nucleophiles with the same nucleophilic atom the effect is small.

One would naively expect this pattern of behaviour to be reproduced by enzymic glycosyl transfer, but the data of Parkin & Schramm (1987) and Stein & Cordes (1981), taken together, apparently indicate behaviour directly at variance with that of the non-enzymic system. Stein & Cordes (1981) used a double-labelling technique to measure $^2\text{H}(V/K)$ for the phosphorolysis of inosine catalysed by *Escherichia coli* purine nucleoside phosphorylase as a function of pH. They interpreted their results in terms of an intrinsic effect in excess of 1.18, masked by isotopically silent steps near the pH optimum. Parkin & Schramm (1987) performed a multiple kinetic isotope effect study of the *N*-glycoside

hydrolysis of AMP catalysed by the AMP nucleosidase of *Azotobacter vinelandii* by using multiple radiolabelling techniques. They found values of $^2\text{H}(V/K)$ of 1.045 and $^3\text{H}(V/K)$ of 1.069 in the absence of the allosteric activator MgATP, and values of 1.030 and 1.047 in its presence. The values in the absence of MgATP were considered to be intrinsic since the enzyme–AMP complex had no commitment to catalysis (DeWolf *et al.*, 1986), and ^{14}N and ^{14}C kinetic isotope effects were high (1.030 and 1.035 respectively).

The reactions catalysed by these two enzymes are very similar: nucleophilic displacement of a purine base from C-1 of a β -ribofuranosyl ring, with inversion of configuration at the reaction centre (Scheme 1). If the enzymic reactions are modelled by the preassociation concerted reactions of $\text{CH}_3\text{OCH}_2\text{--N}^+\text{Me}_2\text{--Ar}$ with nucleophiles, then the DKIEs are the wrong way round, since ionic phosphate is a ‘harder’ nucleophile than water.

Skoog (1986) found a value of $^2\text{H}(V/K)$ of 1.00 for hydrolysis of AMP by AMP nucleosidase, but one of 1.155 for hydrolysis of NMN. He attributed this to isotopically silent steps dominating (V/K) for the good substrate AMP but not for the poor substrate NMN. This interpretation is not compatible with the ^{14}C and ^{15}N isotope effects reported by Parkin & Schramm (1987).

We have therefore remeasured α DKIEs on the purine nucleoside phosphorylase-catalysed phosphorolysis of inosine by using a different technique, that of equilibrium perturbation (Schimerlik *et al.*, 1975). The technique requires that an enzymic reaction is significantly reversible. The position of equilibrium is found by trial and error: the concentration of one participant in the equilibrium is varied until a signal (commonly u.v. absorbance) remains at its original value after the addition of enzyme. The same concentration of isotopically labelled substrate is then used and enzyme is

Abbreviation used: α DKIE, α -deuterium kinetic isotope effect.

† To whom correspondence should be addressed.

Scheme 1. Resemblance of the reactions catalysed by AMP nucleosidase and purine nucleoside phosphorylase to each other and to the reactions of *N*-methoxymethyl-*N,N*-dimethylanilinium ions studied by Knier & Jencks (1980)

added. If there is a kinetic isotope effect, then the forward reaction will proceed faster than the reverse reaction (or vice versa) by virtue of the KIE until the isotope itself achieves its equilibrium distribution. Thus one sees a perturbation in the concentration of the reactants and products. In principle the whole time course of the perturbation could be analysed, but, because it is described by the difference between two exponential terms with closely similar exponents, in practice the accumulation of errors is such that it is best merely to measure the size of the perturbation. In the general case the equations relating the size of the perturbation to the concentrations of reactants are complex, but computer programs are available to solve them (Cleland, 1982b). In the present case it is useful to consider the simpler relationships that arise when there is only a small kinetic isotope effect and no equilibrium effect.

Inosine + phosphate
= hypoxanthine + ribose 1-phosphate

Isotopic label in inosine is retained in ribose 1-phosphate. If there is no equilibrium isotope effect, and the kinetic isotope effect (KIE) is small, then the perturbation (P) in the concentration of inosine is given by:

$$2.72P = \text{KIE} / (1/[\text{inosine}] + 1/[\text{ribose 1-phosphate}])$$

where the KIE is expressed as $\delta k/k$, not k_1/k_2 . Since as soon as there is any reaction after addition of enzyme there is a competition between labelled and unlabelled substrate in both forward and reverse reactions, (V/K) isotope effects are obtained. [If there is no equilibrium isotope effect, then of course (V/K) isotope effects on the forward and reverse reactions must be identical.]

Hitherto equilibrium perturbation has been used only where only one of the participants absorbed. A further reason for performing the present experiments was to explore a system where there is only a small absorbance

change (approx. 20%) between components on either side of the equilibrium.

MATERIALS AND METHODS

Materials

Inosine, adenosine, hypoxanthine and ribose 1-phosphate were bought from the Sigma Chemical Co., Poole, Dorset, U.K.

Purine nucleoside phosphorylase (purine nucleoside:orthophosphate ribosyltransferase) from *Escherichia coli* K12 was purified from a genetically engineered strain as previously described (Cook *et al.*, 1985) except that the last step, Sephadex G-150 column chromatography, was replaced by an affinity chromatography step in which the affinity ligand was Formycin B, which was linked to the matrix as described by Schrader *et al.* (1976). The final preparation obtained was a single band on electrophoresis on an 8% polyacrylamide gel (Ray & Benedict, 1980), and had a specific activity of 90 units ($\mu\text{mol}/\text{min}$)/mg of protein. The enzyme assay method of Kalckar (1947a) as modified by Kim *et al.* (1968) was used. Protein concentration was determined by the Coomassie Blue method (Spector, 1978), with bovine IgG as the reference standard.

Synthesis of [1'- ^2H]inosine

D-[1- ^2H]Ribose was prepared by reduction of D-ribonolactone with sodium amalgam in $^2\text{H}_2\text{O}$. Over a period of 30 min ice-cold solutions of D-ribonolactone (32 g) in $^2\text{H}_2\text{O}$ (50 ml) and 50% (v/v) $^2\text{H}_2\text{SO}_4$ were trickled on to a vigorously stirred mixture of 3% sodium amalgam (618 g) and $^2\text{H}_2\text{O}$ (150 ml) under N_2 , such that the pD of the solution remained between 2.9 and 3.4 and the temperature below 10 °C. After a further hour's

stirring, the mercury was filtered off, and the filtrate was neutralized to pH 5.5 with 0.1 M-NaOH, and concentrated to a fifth of its volume at 35 °C. Na₂SO₄ precipitated by the addition of methanol (500 ml) was filtered off, and the filtrate was evaporated to a yellow syrup (31 g) containing no ribonolactone when examined by t.l.c. The D-[1-²H]ribose was purified via conversion into the *N*-phenylribosylamine sodium sulphate complex (Berger & Lee, 1946), hydrolysis in the presence of benzaldehyde (Berger *et al.*, 1946) and crystallization. It was then converted into 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-[1-²H]ribofuranose by the method of Recondo & Rinderknecht (1959). This material, m.p. 124–129 °C and $[\alpha]_D^{25} + 42.5^\circ$ (*c* 1.5 in chloroform) (lit m.p. 131–132 °C and $[\alpha]_D^{25} + 45.1^\circ$ for the unlabelled compound) was used to ribofuranosylate adenine or hypoxanthine by the method of Vorbrüggen *et al.* (1981), the protocol for hypoxanthine following these authors' protocol for xanthine. The [1'-²H]inosine (13 %) obtained had a ¹H-n.m.r. spectrum identical with that of commercial inosine except for the H-1' signal at δ 5.87 p.p.m. (1H, *J*_{1'-2'} 5.8 Hz) which was only 2 % as intense, and the H-2' signal at δ 4.48 p.p.m., which was a doublet (*J*_{2'-3'} 4.7 Hz) rather than a broad singlet. The proton-decoupled ¹³C-n.m.r. spectra were identical except for the C-1 resonance at 87.7 p.p.m., only just detectable as a triplet in the spectrum of the deuterated compound. Deuteration of labelled substrate in the desired position is thus essentially complete.

Even after recrystallization, large spurious perturbations were obtained in the equilibrium perturbation experiment with this material. It was therefore purified by preparative h.p.l.c. on a reversed-phase C₁₈ column with water as eluent. Inosine and impurities 1, 2 and 3 had relative retention times of 1, 1.43, 3.66 and 4.14 respectively, with impurities 2 and 3 producing only about 20 % of the signal at 265 nm of the main impurity, 1 (itself of the order of 5 % total material). Impurities 1 and 3 were substrates for purine nucleoside phosphorylase. In order to assign a structure to impurity 1, the synthesis was repeated with unlabelled ribose and the unlabelled material was isolated.

Methods

N.m.r. spectra were obtained for [²H₆]dimethyl sulphoxide solutions, with tetramethylsilane as an internal standard, on a JEOL GX270 instrument, in the Fourier-transform mode.

U.v. absorbance was monitored at 250 nm in a Philips PU8800 u.v./visible spectrophotometer, fitted with a thermostatically controlled cell block maintained at 25.0 °C, as measured by a temperature read-out based on a thermistor in a water-containing cell. Spectra were measured on the same machine.

Kinetic and equilibrium data pertain to 0.2 M concentrations of the following buffering agents, to which NaOH was added to achieve the desired pH: pH 5.0, succinic acid; pH 6.1, Mes; pH 7.3, Mops; pH 8.4, Tricine; pH 9.4, glycine.

The second p*K* of ribose 1-phosphate was determined by potentiometric titration at an ionic strength of 0.1 (NaCl).

Equilibrium constants were determined by adding enzyme to a solution containing all participants in the equilibrium, and varying inosine concentration until there was no change in absorbance at 250 nm. Sub-

millimolar total concentrations of all participants in the equilibrium, except phosphate, were used. A typical experiment involved 0.2 mM-inosine, 0.1 mM-hypoxanthine, 0.5 mM-ribose 1-phosphate and 13 mM-phosphate.

When the position of equilibrium had been found, an identical solution, but with deuterated inosine replacing inosine at the same concentration, as estimated by the u.v. absorbance, was used for the equilibrium-perturbation experiment. Because of the instability of ribose 1-phosphate, a slow linear drift in u.v. absorbance could not be avoided, and so enough enzyme was added to ensure that the size of the perturbation was greater than the change due to this drift.

Kinetic isotope effects were calculated from perturbation sizes and the concentrations of participants in the equilibrium by using the program of Cleland (1982*b*), translated from FORTRAN into BASIC and run on a BBC Master microcomputer; the approximate relationship given for illustrative purposes above was used only to check the result, since the KIEs were quite large.

RESULTS AND DISCUSSION

In Table 1 are set out changes in absorption coefficient at 250 nm, equilibrium constant and αDKIE for phosphorolysis of inosine at various pH values. A typical perturbation is shown in Fig. 1.

Fig. 1. Absorbance changes at 250 nm consequent upon the addition of purine nucleoside phosphorylase (150 μg in 5 μl) to a solution (3 ml) containing inosine (0.199 mM), hypoxanthine (0.100 mM), ribose 1-phosphate (0.50 mM) and inorganic phosphate (0.0115 M) in 0.2 M-glycine/NaOH buffer, pH 9.4, in a 1 cm-path-length cell

Table 1. αDKIE measurements of the purine nucleoside phosphorylase-catalysed phosphorolysis of inosine

pH	<i>K</i> _{eq.}	Δ <i>ε</i> (250 nm)	αDKIE*	αDKIE†	Error†	<i>V</i> _{rel.}
5.0	186	916	1.139	1.095	0.030	0.19
6.1	98	1069	1.105	1.100	0.001	0.72
7.3	45.7	1169	1.094	1.008	0.001	1.00
8.4	47.3	1538	1.090	1.156	0.004	0.22
8.4	46.4		1.078			
9.4	45.9	2475	1.164	1.180	0.019	0.041

* Present work.

† Stein & Cordes (1981).

It is clear that meaningful kinetic-isotope-effect data can be obtained by the equilibrium-perturbation technique even if only comparatively small absorption-coefficient differences are monitored. The reproducibility of the data obtained can be judged from the two results at pH 8.4, obtained in completely different sets of experiments. For each set of experiments the KIE was calculated from the best-shaped perturbation of a series: we give no statistical estimates of error, since the main sources of error in equilibrium-perturbation experiments are systematic.

The values of the equilibrium constant and α DKIE and a source of spurious perturbations are discussed below.

Equilibrium constant

There has been considerable confusion in the literature about the value of this constant at 37 °C, values of 54 (Kalckar, 1947b), 3 (Heppel & Hilmoe, 1952), 32 (Tarr, 1958), 40 (de Verdier & Gould, 1963), 57 (Jensen & Nygaard, 1975), 20.8 (Murakami & Tsushima, 1975) and 26 (Salamone *et al.*, 1982) being reported. Some of the confusion arises from studies in which the pH was not controlled. Fig. 2 shows that in fact the equilibrium constant varies with pH in the way expected from the differing pK_2 values of phosphoric acid and ribose 1-phosphate. The infinite-dilution pK_2 of phosphoric acid (7.2) decreases with ionic strength and at an ionic strength of 0.1 is around 6.8 (Bates & Acree, 1943). We find the pK_2 of ribose 1-phosphate, whose infinite-dilution pK_2 is probably close to that of glucose 1-phosphate (6.5; Ashby *et al.*, 1955), to be 6.2 in 0.1 M-NaCl.

The values reported by Salamone *et al.* (1982) and by Murakami & Tsushima (1975) are therefore probably consistent with each other, since the value of 26 pertains to pH 7.0 and ionic strength around 0.02 whereas the value of 21 pertains to pH 7.5 and 0.1 M-Tris/HCl buffer.

The ionizations of the amide-like N-H groups of inosine and hypoxanthine are governed by closely similar pK_a values [8.82 (Albert, 1953) and 8.94 (Albert & Brown, 1954)] and so this ionization will not greatly affect observed equilibrium constants.

α -Deuterium kinetic isotope effects

From the data in Table 1 it is clear that there is broad agreement between the results from equilibrium perturbation and the results reported by Stein & Cordes (1981). The most important difference between the two

sets of results is that we find that the effect is not zero even at pH 7.3. However, the data of Stein & Cordes on the pH variation of the enzyme activity indicate that at pH 7.3 the activity is only about 30 % greater than at pH 6.1, where the isotope effect was at least half expressed. In the limited area of disagreement, therefore, the equilibrium-perturbation data are more compatible with the pH-activity profile than are the results reported by Stein & Cordes (1981).

An intrinsic α DKIE of 15–20 % is reasonably close to α DKIEs measured for hydrolysis of some glycosyl-pyridinium ions by 'retaining' glycoside hydrolases, for example β -galactosidase (Sinnott & Withers, 1974) or α -glucosidase (Hosie & Sinnott, 1985). Since retaining glycosidases invariably possess an active-site carboxylate group (Sinnott, 1987), it appears that displacement of a nitrogen leaving group from the anomeric position of a sugar by an ionized oxygen nucleophile can involve similar transition states, independently of whether the oxygen nucleophile is a protein carboxylate group or the second substrate, phosphate.

The intrinsic effect (1.155) measured by Skoog (1986) for displacement of nicotinamide by a nucleophilic water molecule during hydrolysis of NMN by AMP nucleosidase is also in the same range. It must be that for this enzyme a change of leaving group can dramatically alter α DKIEs, so that they are high with nicotinamide and low with adenosine.

The N-3 isomer of inosine is a substrate

The major impurity in the sample of inosine synthesized by the method of Vorbrüggen *et al.* (1981) must contain a fissile ribofuranosyl-purine bond since it is a substrate for purine nucleoside phosphorylase, and it is likely that the anomeric configuration is β . This is confirmed by the splitting of the H-1' resonance (see below). The compound is therefore inosine β -ribofuranosylated on N-9, N-1, N-3 or O-6 (see Scheme 2). It is not the N-7 isomer, since observed ^{13}C chemical shifts (162.0, 146.8, 145.0, 140.4, 115.0, 92.7, 86.4, 72.8, 70.4 and 61.5 p.p.m.) differ from those reported for this isomer (157.7, 154.1, 144.8, 142.5, 114.8, 89.5, 85.5, 75.1, 69.8 and 61.1 p.p.m.; Chenon *et al.*, 1975). The u.v.-absorption spectrum (λ_{max} , 265 nm, λ_{min} , 230 nm) resembles that reported for the N-3 isomer (λ_{max} , 265 nm, λ_{min} , 232 nm) (Wolfenden *et al.*, 1966), as does the ^1H -n.m.r. spectrum: Tindall *et al.* (1972) report H-8 s, δ 8.67 p.p.m., H-2 s, δ 8.32 p.p.m., and H-1' d,

Fig. 2. Equilibrium constant for phosphorolysis of inosine at 25 °C at I approx. 0.1

The line is that calculated for pH variation with pK_2 values of 6.8 for inorganic phosphate and 6.2 for ribose 1-phosphate.

Scheme 2. Possible sites of attachment of a ribofuranosyl residue to hypoxanthine in a purine nucleoside phosphorylase-labile inosine isomer

δ 5.98 p.p.m., J_{1-2} 6.0 Hz; we find H-8 s, δ 8.59 p.p.m., H-2 s, δ 8.25 p.p.m., and H-1' d, δ 5.87 p.p.m., J_{1-2} 6.4 Hz. *O*-6- and *N*-1-alkylated hypoxanthines exhibit maximal u.v. absorbance at 252 nm (Mason, 1954; Sekiya & Suzuki, 1975). Impurity 1 is therefore 3-*N*-(β -D-ribofuranosyl)hypoxanthine.

Chemical prejudice suggests that a purine base must be protonated before it can depart in a nucleophilic displacement at the anomeric centre of a sugar (see, e.g., Lönnberg & Lehtikoinen, 1982, and references cited therein). Since the acid *pK* values of purine nucleosides are around 2, an enzyme working near neutrality must operate by partial, rather than complete, proton transfer. The position of any acid-catalytic group must therefore be defined, relative to the ribofuranose ring. It is then difficult to see how such a group could protonate both *N*-9-linked and *N*-3-linked purines, yet inosine and its *N*-3 isomer are comparably good substrates.

The possibility thus arises that this enzyme operates without acid catalysis. The departure of pyrimidine anions from 2-deoxyribofuranosyl residues in S_N1 , rather than *A*-1, reactions is known (Shapiro & Kang, 1969), and proton transfer appears to be of minimal importance in the action of some *O*-glycoside hydrolases (Sinnott, 1987).

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