β -Deuterium kinetic isotope effects in the purine nucleoside phosphorylase reaction

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1. [2'-²H]Inosine was made from inosine by tetraisopropylidisiloxanyl protection of the 3'- and 5'-positions, oxidation with dimethyl sulphoxide and acetic anhydride, immediate NaB²H₄ reduction of the oxo sugar product and inversion at C-2' of the resultant protected [2'-²H]arabino-inosine by trifluoromethanesulphonylation and reaction with caesium propionate, followed by deprotection. 2. The equilibrium-perturbation technique was used to measure $\beta^2\text{H}(V/K)$ for phosphorolysis of this compound by the purine nucleoside phosphorylase of *Escherichia coli* as a function of pH. 3. The pH variation indicates an intrinsic effect of 1.06g masked by isotopically silent steps near the pH optimum. 4. The similar pH variation of these β -deuterium effects and the α -deuterium effects measured previously [Stein & Cordes (1981) *J. Biol. Chem.* **256**, 767–772; Lehtikoinen, Sinnott & Krenitsky (1989) *Biochem. J.* **257**, 355–359] for this reaction provides the first experimental reassurance for the common assumption that pH changes merely mask and unmask the chemical steps in an enzyme-catalysed reaction, and do not detectably alter transition-state structure. 5. The dihedral angle between the C–H-2' bond and the electron-deficient *p*-orbital at the transition state is in the range 32–48°, in accord with an essentially planar furanose ring.

INTRODUCTION

Since isotopic substitution does not alter the potential-energy surface for a reaction, kinetic isotope effects are uniquely informative about reaction mechanisms and the structure of transition states. The interpretative difficulties encountered with many other probes in the physical organic chemist's armoury, such as temperature and solvent effects, when they are applied to enzyme reactions make kinetic isotope effects particularly valuable in this area. It is often found, however, that, especially near the pH optimum of the enzyme, intrinsic isotope effects are masked by isotopically silent steps, and it has become common practice to measure isotope effects at a range of pH values. The study by Stein & Cordes (1981) is representative: the authors measured $\alpha^2\text{H}(V/K)$ for the phosphorolysis of inosine by *Escherichia coli* purine nucleoside phosphorylase as a function of pH, and interpreted their results in terms of an intrinsic isotope effect in excess of 1.18 masked by isotopically silent steps near the pH optimum. Later Lehtikoinen *et al.* (1989), prompted by a different pattern of α -deuterium kinetic isotope effects for the enzymic hydrolysis and phosphorolysis of purine nucleosides, and the apparently chemically similar preassociation reactions of *NN*-dimethylmethoxymethylanilinium ions (Knier & Jencks, 1980), redetermined the α -deuterium kinetic isotope effects of the phosphorolysis of inosine using the equilibrium-perturbation method (Schimerlik *et al.*, 1975; Cleland, 1982). They were able to confirm the main features of the behaviour observed by Stein & Cordes (1981).

α -Deuterium kinetic isotope effects have their origin in a weakening of a C–L (L = ¹H or ²H) bending vibration as the hybridization of the carbon atom changes from *sp*³ to *sp*² (Streitwieser *et al.*, 1958; Melander & Saunders, 1980, pp. 172–174). In the absence of an incoming nucleophile, the effect can be used as a measure of the degree of progress towards *sp*² hybridization at the transition state; however, the existence of even a pre-associated nucleophile reduces the effect by obstructing the bending motion of the C–L bond and thus effectively

increasing the force constant for it. Although the variation of α -deuterium kinetic isotope effects with the chemical nature of the nucleophile has been noted on a number of occasions (e.g. Craze *et al.*, 1978), only the study by Knier & Jencks (1980) is extensive enough to establish the possible extent and nature of the variation. In the reactions of *NN*-dimethylmethoxymethylanilinium ions with nucleophiles, β_{ig} and β_{nuc} values establish that the transition state is 'exploded', with bonding to the nucleophile only weakly developed and bonding to the leaving group largely abolished. Nonetheless, measured α -deuterium kinetic isotope effects per deuterium atom vary from 0.99 for F[−] ion to 1.18 for I[−] ion as nucleophiles: even for nucleophiles of the same nucleophilic atom, measurable changes were detectable (e.g. from 1.11 for water to 1.07 for acetate).

It is thus apparent that small changes in transition-state structure, particularly if they are manifested in small changes of the degree of closeness of approach of a preassociated nucleophile, should be manifested in changes of a deuterium kinetic isotope effects more readily than in other isotope effects, or in any measurable structure–reactivity parameters.

Because of the multiplicity of ionizable groups on an enzyme, change of pH will alter the ionic atmosphere at the surface of an enzyme and even to some extent at the active site. There will also be minor changes in protein conformation. Either of these types of change could in principle affect transition-state structure, even without ionization of the active-site groups themselves. Some portion of the changes of α -deuterium effects with pH measured by Stein & Cordes (1981) and Lehtikoinen *et al.* (1989) could have arisen from such changes rather than from a change in rate-determining step, particularly in the light of the sensitivity of this particular type of effect to the precise details of the transition-state structure. The recent reports of different intrinsic primary isotope deuterium effects for the reaction catalysed by yeast, spinach and *E. coli* glutathione reductases (4.3, 2.7 and 1.6 respectively; Vanoni *et al.*, 1990), and the detectably different β_{ig} values for reactions catalysed by experimental evolants of *ebg* β -galactosidases of *E. coli*, differing by only one non-catalytic

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amino acid residue (Li *et al.*, 1989), represent transition-state structure changes consequent upon protein structure changes not obviously greater than the deprotonation of a large number of ionizable groups. The phosphorolysis of inosine catalysed by *E. coli* purine nucleoside phosphorylase is therefore a useful system in which to examine the common assumption that transition-state structure does not alter as the pH is changed.

To do this, the pH variation of another kinetic isotope effect that measures different properties of the transition state, which are less sensitive than α -deuterium kinetic isotope effects to minor structural changes, should be measured. The kinetic effect of β -deuterium substitution (i.e. at the 2'-position of the ribose ring) is suitable. β -Deuterium kinetic isotope effects have their origin in two phenomena, hyperconjugation and the inductive effect of deuterium, and can be considered in terms of eqn. (1) (Melander & Saunders, 1980, pp. 174–180):

$$\ln(k_{1H}/k_{2H}) = \cos^2\theta \cdot \ln(k_{1H}/k_{2H})_{\max} + \ln(k_{1H}/k_{2H})_i \quad (1)$$

The first term represents hyperconjugation of the C–L σ -orbital with an electron-deficient p -orbital on an adjacent carbon atom; θ is the dihedral angle between the C–L bond and this orbital. $\ln(k_{1H}/k_{2H})_{\max}$ is the maximal hyperconjugative effect obtained when the C–L bond and the p -orbital are exactly eclipsed, and increases as the positive charge on the adjacent carbon atom increases, with the associated weakening of the C–L bond.

The second term in eqn. (1) represents a small geometry-independent inductive deuterium effect. It is found experimentally that deuterium has apparently a small electron-donating inductive effect, but there is no consensus as to its theoretical origin (one view being that the anharmonicity of the C–L bond vibration, even in the zeroth vibrational state, results in the centre of electron density of the C–L σ -bond lying slightly more towards the carbon atom in the case of the C– 2 H bond than the C– 1 H bond).

The β -deuterium kinetic isotope effect on the enzyme-catalysed phosphorolysis of inosine should therefore measure electronic and conformational properties of the transition state much less sensitive to local protein environment than the α effect.

METHODS AND MATERIALS

General methods

Buffer solutions and enzyme have been described previously (Lehikoinen *et al.*, 1989).

Equilibrium perturbations were measured by monitoring the change in u.v. absorbance with a Perkin–Elmer Lambda 6 u.v.–visible spectrometer, fitted with the manufacturer's Peltier-effect thermostatically controlled cell block, which maintained temperature to within ± 0.1 °C. Initial experiments at pH 9.4 were performed exactly analogously to those described by Lehikoinen *et al.* (1989) (who made a transcriptional error: the perturbation displayed in Fig. 1 of that paper was obtained in a 0.5 cm-pathlength cell, not a 1.0 cm-pathlength cell). However, the smaller β effects made it impossible to use those wavelengths used by Lehikoinen *et al.* (1989), which were the wavelengths of maximal absolute absorption-coefficient change, at other pH values. We instead chose the wavelength of greatest relative absorption-coefficient change, 280 nm, and adjusted the concentrations of the reagents so as to have an absorbance of about 1. $\Delta\epsilon$ values were $916 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 5.0, $896 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 6.1, $860 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 7.3 and $732 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 8.4, there being an increase in absorbance on phosphorolysis of inosine. Kinetic isotope effects were calculated from observed perturbations by

using the program of Cleland (1982), run on an Epson Equity III microcomputer.

The equilibrium constants for the phosphorolysis of [2'- 2 H]inosine obtained from the equilibrium-perturbation measurements were within 1–3 % of those obtained previously (Lehikoinen *et al.*, 1989), with the exception of the value at pH 7.3, for which we now report a value of 67 instead of 45.7. This value in fact fits the theoretical pH variation deduced by Lehikoinen *et al.* (1989) better than the original value. We suspect that the error arose from the instability of ribose 1-phosphate: under the conditions of the experiment a 10 % decomposition to inorganic phosphate would have accounted for the discrepancy.

Synthesis of [2'- 2 H]inosine

1 H-n.m.r. spectra were obtained on a Bruker AM 400 instrument at 400 MHz.

O-3',5'-(1'',1'',3'',3''-Tetraisopropylidisiloxanyl)inosine. To dried inosine (4.0 g) suspended in dry pyridine (150 ml) was added 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane (5.35 ml) in dry pyridine (50 ml), and the mixture was stirred at room temperature for 7 days, by which time the inosine had dissolved. The pyridine was then evaporated under reduced pressure and the residue was partitioned between ethyl acetate and water. The organic phase was washed with aq. 1 M-HCl (2×100 ml), water, saturated NaHCO_3 solution and saturated NaCl solution, dried with anhydrous Na_2SO_4 and evaporated. The residue was chromatographed on a 5.5 cm $\times$ 20 cm column of Merck grade 60 silica gel with chloroform followed by chloroform/methanol (30:1, v/v) for elution of the product (6 g, 75 %) as a gum. 1 H n.m.r.: δ (p.p.m.) ($\text{d}[^2\text{H}_3]$ methyl sulphoxide) 12.31 (1H, bs, base N–H), 8.19 (1H, s, base C–H), 8.02 (1H, s, base C–H), 5.97 (1H, d, H-1'), 5.72 (1H, bs, 2'-OH) 4.45 (1H, m, H-2'), 4.62 (1H, m, H-3'), 4.04 (1H, m, H-4'), 4.08 (1H, m, H-5'_a'), 4.10 (1H, m, H-5'_b') and 0.90–1.10 (28H, m, isopropyl C–H).

O-3',5'-(1'',1'',3'',3''-Tetraisopropylidisiloxanyl)[2'- 2 H]inosine and 9-{3',5'-O-(1'',1'',3'',3''-tetraisopropylidisiloxanyl)- β -D-[2'- 2 H]arabinofuranosyl}hypoxanthine. 3',5'-O-(1'',1'',3'',3''-Tetraisopropylidisiloxanyl)inosine (1.021 g, 2 mmol) was dissolved in dry dimethyl sulphoxide (8.0 ml), and acetic anhydride (1.0 ml) was added: the solution was stirred under N_2 for 24 h, then tetrahydrofuran (40 ml) was added and the mixture was cooled to -12 °C. A solution of NaB^2H_4 (1.0 g) in aq. 50 % (v/v) propan-1-ol (30 ml) was added over a period of 20 min with stirring. Stirring was continued for a further 30 min, then saturated NaCl solution (30 ml) was added. The solution was extracted with ethyl acetate, and the organic phase was washed with saturated NaCl solution, dried with anhydrous Na_2SO_4 and evaporated under vacuum. The residue was applied to a 2.5 cm $\times$ 20 cm column of silica gel, and eluted first with chloroform and then with chloroform/methanol (30:1, v/v). 3',5'-O-(1'',1'',3'',3''-Tetraisopropylidisiloxanyl)[2'- 2 H]inosine (30 mg) was eluted first, followed by 9-{3',5'-O-(1'',1'',3'',3''-tetraisopropylidisiloxanyl)- β -D-[2'- 2 H]arabinofuranosyl}hypoxanthine (680 mg, 67 %). 1 H n.m.r.: δ (p.p.m.) ($\text{d}[^2\text{H}_3]$ methyl sulphoxide) 12.30 (1H, bs, base N–H), 8.01 (2H, s, coincident base C–H), 6.18 (1H, s, H-1'), 5.81 (1H, bs, 2'-OH), 4.53 (1H, d, H-3'), 4.04 (1H, m, H-4'), 4.10 (1H, m, H-5'_a'), 4.08 (1H, m, H-5'_b') and 0.90–1.10 (28H, m, isopropyl C–H).

O-9-[2'-Trifluoromethanesulphonyl-3',5'-(1'',1'',3'',3''-O-tetraisopropylidisiloxanyl)- β -D-[2'- 2 H]arabinofuranosyl}hypoxanthine. To 9-{3',5'-O-(1'',1'',3'',3''-tetraisopropylidisiloxanyl)- β -D-[2'- 2 H]arabinofuranosyl}hypoxanthine (1.022 g, 2 mmol) and 4-(*NN*-dimethylamino)pyridine (752 mg, 7.07 mmol) in dry dichloromethane (50 ml) was added dried pyridine (1.3 ml, 15.9 mmol). The solution was cooled to 0 °C, and trifluoromethanesulphonic

anhydride (0.52 ml, 3.10 mmol) was added over a period of 10 min. The mixture was then stirred for 2 h at 0 °C, then poured into cold saturated NaHCO₃ solution (50 ml). The organic layer was separated, and the aqueous layer was further extracted with dichloromethane (2 × 50 ml). The combined organic layers were dried over MgSO₄ and evaporated to give a syrup, which was purified by chromatography on a 2.5 cm × 20 cm silica gel column, with chloroform followed by chloroform/methanol (40:1, v/v) as eluent. The yield was 650 mg (50%). ¹H n.m.r.: δ (p.p.m.) ([²H]chloroform) 8.18 and 8.04 (1H and 1H, ss, base C-H), 6.43 (1H, s, H-1'), 5.22 (1H, d, H-3'), 4.02–4.34 (3H, m, H-4' and H-5') and 0.90–1.20 (28H, m, isopropyl C-H).

O-2'-Propionyl-3',5'-O-(1'',1'',3'',3''-tetraisopropylidisiloxanyl)-[2'-²H]inosine. To a stirred solution of 9-[2'-O-trifluoromethanesulphonyl-3',5'-(1'',1'',3'',3''-O-tetraisopropylidisiloxanyl)- β -D-[2'-²H]arabinofuranosyl]hypoxanthine (644 mg, 1 mmol) in dry dimethylformamide (2.5 ml) at room temperature was added anhydrous caesium propionate (273 mg, 1.32 mmol). The mixture was stirred 4 h at room temperature, then the dimethylformamide was evaporated and the residue was partitioned between water and ethyl acetate, with back-extraction of the aqueous layers with fresh ethyl acetate. The ethyl acetate was extracted with saturated NaCl solution, dried with anhydrous Na₂SO₄ and evaporated to yield a white solid, which was purified by column chromatography, as described above. The yield was 90%. ¹H n.m.r.: δ (p.p.m.) ([²H]chloroform) 8.14 and 8.08 (1H and 1H, ss, base C-H), 6.05 (1H, s, H-1'), 4.95 (1H, d, H-3'), 4.02–4.34 (3H, m, H-4' and H-5'), 2.5 (2H, m, propionate CH₂) and 0.90–1.20 (31H, m, isopropyl and propionate methyl).

[2'-²H]Inosine. To a solution of 2'-O-propionyl-3',5'-O-(1'',1'',3'',3''-tetraisopropylidisiloxanyl)[2'-²H]inosine (291 mg, 0.5 mmol) in tetrahydrofuran (20 ml) was added a 1.0 M solution of tetrabutylammonium fluoride in the same solvent (1.0 ml, 2 equiv.). The mixture was stirred overnight at room temperature, then conc. aq. NH₃ (3.0 ml) was added, and the mixture was stirred for a further 24 h. The solvent was evaporated, and the yellow residue was partitioned between water (30 ml) and dichloromethane (25 ml). The dichloromethane layer was back-extracted with water, and the combined aqueous layers were evaporated and purified by chromatography on a 2.5 cm × 20 cm

column of silica gel with chloroform/methanol (8:1, v/v) as eluent. The yield was 94 mg (70%). ¹H n.m.r.: δ (p.p.m.) (di[²H₃]methyl sulphoxide) 12.4 (1H, bs, base N-H), 8.33 and 8.07 (1H and 1H, ss, base C-H), 5.96 (1H, s, H-1'), 5.47, 5.36 and 5.28 (1H, 1H and 1H, bss, 2'-, 3'- and 5'-O-H), 4.12 (1H, d, H-3') and 3.65 (1H, m, H-4').

For enzyme experiments the material was further purified by semi-preparative h.p.l.c. (RP-18 column, 9 ml/min isocratic elution with water) on a Spectrophysics SP8800 system.

RESULTS AND DISCUSSION

Synthesis of [2'-²H]inosine

The synthesis of [2'-²H]inosine from inosine was achieved with conventional chemistry, as depicted in Scheme 1. Precedent for the use of tetraisopropylidisiloxanyl protection of the 3'- and 5'-positions was Robins & Wilson (1983), that for the dimethyl sulphoxide/acetic anhydride oxidation of the 2'-hydroxy group and immediate NaBH₄ reduction of the unstable 2'-oxonucleoside was Hansske *et al.* (1984), and that for the inversion of the 2'-stereochemistry of the *arabino*-inosine product by trifluoromethanesulphonylation and S_N2 displacement by caesium propionate was Jiang *et al.* (1988).

The p.m.r. spectrum of the 2'-deuterated product was identical with that of commercial inosine, except that the H-2' signal at δ 4.48 p.p.m. had disappeared and the H-1' signal at δ 5.84 p.p.m. was a singlet rather than a doublet.

Equilibrium-perturbation measurements

Fig. 1 shows the perturbation observed with [2'-²H]inosine at pH 9.4, in the exact analogue of the experiment of Lehtikoinen *et al.* (1989) with [1'-²H]inosine. Table 1 gives the derived values of $\beta^{2H}(V/K)$ from this and experiments at other pH values, together with the values of $\alpha^{2H}(V/K)$ from Lehtikoinen *et al.* (1989) and Stein & Cordes (1981). All values of $\beta^{2H}(V/K)$ except that at pH 9.4 are the results of duplicate sets of experiments.

Scheme 1. Synthesis of [2'-²H]inosine

Reagents: a, 1,3-dichloro-1,1,3,3-tetraisopropylidisiloxane in pyridine; b, acetic anhydride in dimethyl sulphoxide; c, NaBH₄; d, trifluoromethanesulphonic anhydride, 4-(*NN*-dimethylamino)pyridine in dichloromethane; e, caesium propionate in dry dimethylformamide; f, tetrabutylammonium fluoride in tetrahydrofuran; g, conc. aq. NH₃.

Fig. 1. Absorbance changes at 250 nm consequent upon addition of purine nucleoside phosphorylase (E) (5 μ l, 120 μ g) to a solution (1.5 ml) containing [2'- 3 H]inosine (0.200 mM), hypoxanthine (0.100 mM), ribose 1-phosphate (0.500 mM) and inorganic phosphate (0.0115 M) in 0.2 M-glycine/NaOH buffer, pH 9.4, in a 0.5 cm-pathlength cell at 25.0 $^{\circ}$ C

Table 1. Secondary deuterium kinetic isotope effects on the phosphorolysis of inosine by *E. coli* purine nucleoside phosphorylase at 25.0 $^{\circ}$ C

pH	α -Deuterium kinetic isotope effect*	β -Deuterium kinetic isotope effect
5.0	1.139	1.063
6.1	1.105	1.056
7.3	1.094	1.036
8.4	1.090	1.043
9.4	1.164	1.068

* Values from Lehtikoinen *et al.* (1989).

Interpretation of the pH variation of α - and β -deuterium kinetic isotope effects

The equilibrium-perturbation technique yields kinetic isotope effects on V/K (Schimerlik *et al.*, 1975; this can be appreciated intuitively from the consideration that immediately after the addition of enzyme there is labelled substrate competing with unlabelled substrate on one side of the equilibrium, and labelled product competing with unlabelled product on the other). The general form of the expression for isotope effects on V/K is given by eqn. (2) (Northrop, 1981):

$$^2H(V/K) = (^2Hk + C_f + C_r \cdot ^2HK)/(1 + C_f + C_r) \quad (2)$$

where 2Hk is the intrinsic isotope effect on the isotope-sensitive step, 2HK is the isotope effect on the position of equilibrium, and C_f and C_r are the forward and reverse commitments to catalysis respectively. C_f represents the tendency of the enzyme complex immediately before the isotope-sensitive step to continue forward as opposed to its tendency to partition back to free enzyme and substrate, whereas C_r represents the tendency of the first enzyme complex after the isotope-sensitive step to undergo the reverse of this step rather than go on to products.

If the kinetic isotope effect is small, $^2H(V/K)$ and 2Hk can be written $1 + \delta_{\text{obs}}$ and $1 + \delta_{\text{in}}$ respectively. In the case of the phosphorolysis of inosine, the charge on the ribofuranose ring and hybridization of the carbon atoms in it is the same in the substrate and in the product. It is therefore likely that equilibrium isotope effects will be small compared with kinetic isotope effects,

so that 2HK can be set equal to unity. Eqn (3) should then hold for both types of effect:

$$\delta_{\text{obs}} = \delta_{\text{in}}/(1 + C_f + C_r) \quad (3)$$

As the pH is changed, if only the commitments alter, then the ratio of δ_{obs} values for two different types of small kinetic isotope effect should be constant and equal to the ratio of the δ_{in} values. If on the other hand transition-state structure alters, particularly as a consequence of the closeness of approach of a pre-associated nucleophile, then there should be a systematic variation of the ratio of the two δ_{obs} values with pH.

The ratio of the δ_{obs} values for α - and β -deuterium kinetic isotope effects for the phosphorolysis of inosine, when both effects are measured by the equilibrium-perturbation method, is found to be 2.2 at pH 5.0, 2.0 (average) at pH 6.1, 2.6 at pH 7.3, 2.1 and pH 8.4 and 2.4 at pH 9.4.

It is clear that there is no systematic variation of this ratio with pH, the observed variation being within experimental error. We therefore conclude that for this enzymic reaction, which chemical precedent suggests might have a transition-state structure particularly prone to alteration with change in ionic atmosphere, change of pH merely masks and unmasks a chemical transition state of constant structure.

Conformation of the furanose ring at the transition state

To obtain a value of θ from a single intrinsic β -deuterium kinetic isotope effect of 1.068 requires estimates of likely values of $\ln(k_{1H}/k_{2H})_{\text{max}}$ and $\ln(k_{1H}/k_{2H})_i$ (eqn. 1). The data of Kresge & Weeks (1984) on secondary deuterium isotope effects on the hydrolysis of acetaldehyde diethyl acetal provide a basis for estimating these, and for obtaining a lower limit of θ . In water, the hydrolysis is specifically acid-catalysed and involves the ethoxyethyl cation as a real intermediate. The α -deuterium effect is 1.176 ± 0.019 , within experimental error the same as the intrinsic effect for the phosphorolysis of inosine measured by Lehtikoinen *et al.* (1989). The presence of a pre-associated nucleophilic phosphate will, however, result in an α -deuterium kinetic isotope effect lower than that for a true S_N1 reaction with an equal degree of charge development at the transition state. Therefore the estimates of $\ln(k_{1H}/k_{2H})_{\text{max}}$ and $\ln(k_{1H}/k_{2H})_i$ obtained from the hydrolysis of acetaldehyde diethyl acetal will be lower limits for the enzyme-catalysed phosphorolysis of inosine.

The inductive effect of α -deuterium substitution can be estimated from the isotope effect on the hydrolysis of $\text{CH}_2=\text{CL}-\text{OEt}$; the rate-determining step is the protonation of the methylene carbon atom, but as there is no change of hybridization at the carbocationic carbon atom the effect (0.965) is wholly inductive. Inductive effects are attenuated by approx. 2.5-fold per additional carbon atom between the reaction centre and the site of substitution (e.g. Lowry & Richardson, 1981, p. 139), so we take $\ln(k_{1H}/k_{2H})_i$ as -0.014 . The hyperconjugative β -deuterium kinetic isotope effect of a freely rotating methyl group is $\frac{3}{2} \cdot \ln(k_{1H}/k_{2H})_{\text{max}}$ (Hehre, 1975). From the experimental β -deuterium kinetic isotope effect for the hydrolysis of $\text{CL}_3\text{-CH(OEt)}_2$ (1.134 ± 0.011) and a value of $-0.014 \ln(k_{1H}/k_{2H})_i$, we can estimate $\ln(k_{1H}/k_{2H})_{\text{max}}$ as 0.112. Substituting values of 0.112 for $\ln(k_{1H}/k_{2H})_{\text{max}}$ and -0.014 for $\ln(k_{1H}/k_{2H})_i$, and the experimental intrinsic β -deuterium kinetic isotope effect for phosphorolysis of inosine, yields a value of θ of 32° , which will be a lower limit, but, because of the chemical similarity of the model reaction, probably a close one.

An upper limit on θ can be obtained by using the data of Siehl & Walter (1984) on the effect of β -deuterium substitution on the thermodynamic stability of a fully formed simple carbocation, observed under stable ion conditions: at 25 $^{\circ}$ C the 2-methyl-3- $^3\text{H}_3$ methylbicyclo[2.2.2]octan-2-yl cation is favoured over

the 2-[$^2\text{H}_3$]methyl-3-methylbicyclo[2.2.2]octan-2-yl cation by 589 kJ/mol. On combination of this datum with a similarly extreme estimate of $\ln(k_1/k_2)$ of -0.041 obtained by calculation for gas-phase reactions (Williams, 1985), then an estimate of θ of 48° for the enzymic reaction results. This is a remote upper limit.

The high intrinsic α -deuterium kinetic isotope effect for the enzymic reaction suggests that C-1' has essentially sp^2 hybridization at the transition state. If indeed this is the case, then a fully planar furanose ring would give a value of θ of 30° . This would increase if C-3' moved to below the plane of the ring, towards the ^3E conformation. This would also have the effect of removing the 3'-hydroxy group out of the likely trajectory of the incoming nucleophilic phosphate.

We therefore conclude that, at the transition state for phosphorolysis of inosine, the ribose ring is essentially planar, with perhaps a small amount of pucker towards the ^3E conformation. Published X-ray crystal structures of nucleoside phosphorylase appear not yet to have the resolution necessary to bear upon the transition-state conformation. Although the structure of the *E. coli* purine nucleotide phosphorylase is not yet available, the structure of the enzyme from human erythrocytes appears to indicate that one oxygen atom of the bound phosphate group is poised for the formation of a covalent bond to the anomeric centre, approaching from the opposite side to the departing base (Ealick *et al.*, 1990). However, the structure of the thymidine phosphorylase crystallized from *E. coli* is such that the nucleoside-binding site and apparent phosphate-binding site are 1.0 nm apart (Walter *et al.*, 1990), indicating at the very least a remarkable conformational flexibility of the enzyme.

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