Carbon-13 and Deuterium Isotope Effects on the Reaction Catalyzed by Glyceraldehyde-3-phosphate Dehydrogenase†

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Received May 1, 1991; Revised Manuscript Received June 19, 1991

ABSTRACT: Carbon-13 and deuterium isotope effects have been measured on the reaction catalyzed by rabbit muscle glyceraldehyde-3-phosphate dehydrogenase in an effort to locate the rate-limiting steps. With D-glyceraldehyde 3-phosphate as substrate, hydride transfer is a major, but not the only, slow step prior to release of the first product, and the intrinsic primary deuterium and ^{13}C isotope effects on this step are 5–5.5 and 1.034–1.040, and the sum of the commitments to catalysis is ~3. The ^{13}C isotope effects on thiohemiacetal formation and thioester phosphorolysis are 1.005 or less. The intrinsic α-secondary deuterium isotope effect at C-4 of the nicotinamide ring of NAD is ~1.4; this large normal value (the equilibrium isotope effect is 0.89) shows tight coupling of hydrogen motions in the transition state accompanied by tunneling. With D-glyceraldehyde as substrate, the isotope effects are similar, but the sum of commitments is ~1.5, so that hydride transfer is more, but still not solely, rate limiting for this slow substrate. The observed ^{13}C and deuterium equilibrium isotope effects on the overall reaction from the hydrated aldehyde are 0.995 and 1.145, while the ^{13}C equilibrium isotope effect for conversion of a thiohemiacetal to a thioester is 0.994, and that for conversion of a thioester to an acyl phosphate is 0.997. Somewhat uncertain values for the ^{13}C equilibrium isotope effects on aldehyde dehydration and formation of a thiohemiacetal are 1.003 and 1.004.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[1] catalyzes the oxidation and phosphorylation of D-glyceraldehyde 3-phosphate to yield 1,3-bisphosphoglycerate:

$$NAD + GAP + P_i \rightleftharpoons 1,3\text{-bisphosphoglycerate} + NADH + H^+ \quad (1)$$

The equilibrium constant for this reaction is 2×10^{-8}, so the reaction with phosphate as a substrate is readily reversible at physiological pH. While D-glyceraldehyde is a slow (V_{max} ~ 5%) and poorly bound (K_m 250 times that of glyceraldehyde 3-phosphate) substrate, when phosphate is replaced by arsenate the reaction goes at the same rate but produces 3-phosphoglycerate, since 1-arseno-3-phosphoglycerate hydrolyzes spontaneously.

The kinetic mechanism of the rabbit muscle enzyme at pH 8.6 is ordered with NAD, glyceraldehyde 3-phosphate and phosphate adding in that order and NADH being released last (Orsi & Cleland, 1972). The rate-limiting step with glyceraldehyde 3-phosphate is NADH release, but with 2-deoxyglyceraldehyde 3-phosphate it appears that phosphate reaction has become rate limiting, since V_{max} is 13-fold slower with phosphate but only 2-fold slower with arsenate, while with glyceraldehyde-3-phosphate the V_{max} is the same with both substrates. With glyceraldehyde it appears that a step prior to reaction with phosphate or arsenate is rate limiting, since V_{max} is reduced by almost the same factor with phosphate as with arsenate as a substrate.

The chemical mechanism involves formation of a thiohemiacetal (*S* configuration at C-1) with Cys-149,[2] followed by hydride transfer to the *pro-S* side of the nicotinamide ring of NAD (Moras et al., 1975). His-176 is presumably the acid–base catalyst for these steps, and the log(V/K) profile vs pH for phosphate decreases below the pK of phosphate and what is probably the pK of His-176 at ~6 to a slope of 2 at low pH (see Results). His-176, which should be protonated after hydride transfer, presumably helps catalyze the attack of phosphate or arsenate on the thioester to give the final product. The present work was undertaken to explore the degree to which hydride transfer is rate limiting for the portion of the reaction up to release of the first product and formation of E–NADH. Isotope effects on V/K for phosphate (or on apparent V/K and V for glyceraldehyde 3-phosphate or glyceraldehyde with phosphate at $0.1K_m$, which gives equivalent values) reflect steps only up to the first irreversible one, and thus the slow later release of NADH has no effect on these values. We have measured ^{13}C isotope effects at C-1 with deuterated and unlabeled glyceraldehyde 3-phosphate and glyceraldehyde, as well as the deuterium isotope effect, since this allows estimation of intrinsic isotope effects for the hydride transfer step (Hermes et al., 1982). With glyceraldehyde 3-phosphate, we have also measured the α-secondary deuterium isotope effect at C-4 of the nicotinamide ring of NAD and the effect of deuteration in this position on the ^{13}C isotope effect, since this permits a more exact solution for intrinsic isotope effects. We have also tried to determine ^{13}C equilibrium isotope effects for reactions corresponding to each step in the chemical mechanism. We conclude that hydride transfer is a major rate-limiting step for V/K with both glyceraldehyde 3-phosphate and glyceraldehyde, although slightly more so with the latter. Finite commitments do exist with both substrates, however, so that other steps partly limit the rate.

† Supported by grant GM 18938 from the NIH. P.F.C. held a NIH postdoctoral fellowship (GM 09341).

[1] Abbreviations: GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; Aces, 2-[(2-amino-2-oxoethyl)amino]ethanesulfonic acid.

[2] The numbering is that of the lobster enzyme, for which an X-ray structure is known (Moras et al., 1975).

0006-2960/91/0430-8871$02.50/0

Materials and Methods

NAD-4-*d* was synthesized by incubating in 100 mL of 2.95 mM ethanol-d_6, 5.6 mM NAD, 100 units of liver alcohol dehydrogenase, and 200 units of aldehyde dehydrogenase until the A_{340} reached a maximum (3.4). At this point 500 units of glutamate dehydrogenase and 10 mM α-ketoglutarate were added, and when A_{340} reached 0.09, the solution was passed through an Amicon PM30 untrafilter to remove the enzyme and concentrated at 25 °C to 22 mL. This solution was loaded on a C_{18} reverse-phase preparative HPLC column previously washed with 30 mM potassium phosphate, pH 7.0. The column was eluted with the same buffer, and A_{260} was used to locate nucleotide peaks. GAPDH assays were used to confirm which peak was NAD. The fractions containing NAD-4-*d* (800 mL) were reduced to 20 mL by rotoevaporation at 25 °C.

DL-Glyceraldehyde 3-phosphate-1-*d* (99.3% D) was synthesized by the method of Serianni et al. (1979), which involves lead acetate oxidation of glycerol-3-phosphate, cyanide addition to the resulting glycolaldehyde-phosphate, and hydrogenation of the resulting cyanohydrin with D_2 gas. DL-Glyceraldehyde-1-*d* (99.1% D) was obtained similarly from nonphosphorylated glycerol.

5-Thioglucose 6-phosphate was synthesized by incubating 10 units/mL yeast hexokinase, 4 mM $Mg(OAc)_2$, 100 mM ATP, 30 mM 5-thioglucose, 0.2 mM dithiothreitol, and 100 mM Hepes, pH 8, for 20 h at 25 °C.

^{13}C Isotope Effects. The natural abundance of ^{13}C in the substrates was used as the label, and changes in the $^{13}C/^{12}C$ ratio at C-1 were used to calculate V/K isotope effects. Coupling systems were used to recycle phosphate and to remove NADH and 1,3-bisphosphoglycerate. Low conversion samples (10 mL) contained 50 mM aldehyde substrate, 0.4 mM phosphate, 0.2 mM NAD, 20 mM ADP, 20 mM $Mg(OAc)_2$, 1 mM dithiothreitol, and 50 mM Taps. When the substrate was glyceraldehyde-3-phosphate, 10 mM D-glyceraldehyde was also added. The solution was adjusted to pH 4 and degassed with CO_2-free N_2 overnight. The pH was then adjusted to 8, 9.8 μmol of acetaldehyde was added, and the solution was degassed for 30 min more. Ten units each of alcohol dehydrogenase, 3-phosphoglycerate kinase, and glycerokinase and 2 units of GAPDH were added. The fraction of reaction was controlled at ~0.2 by the amount of acetaldehyde added, which was essential for the regeneration of NAD from NADH. The ATP formed by the 3-phosphoglycerate kinase reaction was hydrolyzed by the ATPase activity of glycerokinase with D-glyceraldehyde. When glyceraldehyde 3-phosphate was the substrate, the D-glyceraldehyde added to permit this ATPase contributed only ~1% of the glycerate formed by the GAPDH-catalyzed oxidation.

After the reaction had proceeded overnight, the reaction mixture was passed through an Amicon ultrafilter to remove enzymes and then through a 1.4 × 4.5 cm column of charcoal (pretreated with HCl and EDTA) to remove nucleotides. After loading the sample, the column was washed with 2 mL of water. When glyceraldehyde 3-phosphate was the substrate, 10 units of alkaline phosphatase were added at this point to hydrolyze 3-phosphoglycerate. The eluate was then loaded onto a 1.5 × 10.5 cm column of Dowex-1-X8 resin in the chloride form. The column was washed with 20 mL of water, and glycerate was eluted with 50 mL of 150 mM KCl in 5 mM phosphate, pH 7. Glycerate elution was followed by testing the fractions for periodate consumption, and those fractions containing glycerate were pooled and concentrated to 5–10 mL.

The concentrated column fractions were placed in a sealed reaction vessel, titrated to pH 3, and purged with CO_2-free N_2 overnight. The pH was then adjusted to 4.5 and a 3-fold molar excess of HIO_4 (adjusted to pH 4.5) was added and the oxidation allowed to proceed for 4 h. This oxidation produces CO_2, formate, and formaldehyde from carbons 1, 2, and 3, with the cleavage between C-2 and C-3 being rapid and the subsequent oxidation of glyoxylate being much slower. Complete oxidation is essential, since the ^{13}C isotope effect at C-1 on this oxidation is ~3%. The remaining periodate was then consumed by adding ethylene glycol equimolar to the original level of periodate. The pH of the solution was adjusted to 1 with sulfuric acid, and CO_2 was isolated on a vacuum line and analyzed in an isotope ratio mass spectrometer.

Complete conversion samples (10 mL) contained 10 mM aldehyde substrate, 10 mM sodium arsenate, 1 mM NAD, 1 mM dithiothreitol, and 50 mM Taps. The solution was degassed and handled as described above, except that no 3-phosphoglycerate kinase or glycerokinase was added, since the reaction is irreversible with arsenate as the substrate because 1-arseno-3-phosphoglycerate spontaneously decomposes. NADH was recycled as usual, however.

When the ^{13}C isotope effect was determined with NAD-4-*d* as substrate, the recycling system was changed by replacing acetaldehyde and alcohol dehydrogenase with 10 mM dihydroxyacetone phosphate and 10 units of glycerol 3-phosphate dehydrogenase. Since this enzyme has the same prochiral specificity as GAPDH, the deuterium remained in NAD during the recycling process, while it would have been removed by alcohol dehydrogenase.

Deuterium Isotope Effects. These isotope effects were obtained by varying the concentrations of racemic 1-deuterated or unlabeled aldehyde substrate at 0.4 mM phosphate ($0.1K_m$) in the presence of 2 mM NAD, 1 mM dithiothreitol, and 50 mM Taps, pH 8.6. To recycle 1,3-bisphosphoglycerate, 20 mM MgADP and 3 units each of 3-phosphoglycerate kinase and glycerokinase were added, and when glyceraldehyde 3-phosphate was the substrate, 10 mM D-glyceraldehyde was added to induce the ATPase activity of glycerokinase. The added D-glyceraldehyde contributed ~0.5% to the measured reaction rate. When DL-glyceraldehyde was the substrate, glycerokinase catalyzed both ATPase with D-glyceraldehyde and phosphorylation of L-glyceraldehyde.

pH Profiles. pH profiles were run in a system containing 2 mM NAD, 0.5 mM glyceraldehyde 3-phosphate, variable phosphate, and a buffer consisting of 100 mM Aces, 50 mM Tris, 50 mM ethanolamine, and 1 mM dithiothreitol. Addition of 20 mM MgADP, 10 mM D-glyceraldehyde and 0.3–3 units/mL of 3-phosphoglycerate kinase and glycerokinase recycled 1,3-bisphosphoglycerate and hydrolyzed the ATP formed as the result of the D-glyceraldehyde-induced ATPase activity of glycerokinase so that the reaction remained linear for 6 min and could be followed spectrophotometrically at 340 nm. From 4–40 milliunits of GAPDH were added to start the reaction.

Data Analysis. Equilibrium isotope effects were calculated from $^{13}C/^{12}C$ ratios in one reactant at equilibrium (R_{eq}) and in the total pool (i.e., starting material, R_o) and the fraction of the total pool that remained as this reactant at equilibrium (f_{eq}):

$$^{13}K_{eq} = [1 + f_{eq}(1 - R_{eq}/R_o)]/(R_{eq}/R_o) \qquad (2)$$

Kinetic isotope effects were determined from the mass ratios in product (R_p) and initial substrate (R_o, normally determined in the product after 100% reaction) and the fractional reaction f:

$$^{13}(V/K) = \log (1 - f)/[\log (1 - fR_p/R_o)] \quad (3)$$

Kinetic deuterium isotope effects were determined by fitting initial velocities to

$$v = VA/[(K + A)(1 + F_I E_v)] \quad (4)$$

where K and V are K_m and V_{max} for unlabeled substrate and A is substrate concentration. F_I is the fraction of deuterium in the substrate and E_v the isotope effect minus 1 on both V and V/K.

pH profiles were fitted to eq 5 when the slope reached 2 on the acid side and to eq 6 when it reached only 1.

$$\log y = \log [c/[1 + H/K_1 + H^2/(K_1K_2)]] \quad (5)$$

$$\log y = \log [c/(1 + H/K_1)] \quad (6)$$

In these equations y is the kinetic parameter, H is $[H^+]$, c is a constant, and K_1 and K_2 are acid dissociation constants.

Results

pH Profiles. Log (V/K_{Pi}) when plotted vs pH decreased to a slope of 2 below two pK's that were too close to determine separately (average value: 6.20 ± 0.05). One of these pK's is that of phosphate, while the other is probably that of His-176, the acid–base catalytic group in the active site. Log V vs pH decreased below a pK of 6.2 ± 0.1 and possibly below a second pK of 4.75 ± 0.25.

To determine the dissociation constants of glyceraldehyde 3-phosphate and glyceraldehyde from E–NAD as a function of pH, the apparent K_m values for these substrates were determined at $0.1K_m$ levels of arsenate (from 0.4–4 mM, with the higher values at lower pH) in a system containing 4 mM NAD and the same buffer as noted above. Under these conditions, the apparent K_m of the aldehyde substrate is its dissociation constant. The K_i for glyceraldehyde 3-phosphate determined in this way was ~1 mM at high pH but decreased below a pK of 7.0 ± 0.1 (it became 30 μM at pH 5.53). This pK is probably that of glyceraldehyde 3-phosphate itself, since the K_i for glyceraldehyde determined in the same fashion was pH independent at ~225 mM.

Equilibrium Isotope Effects. Glyceraldehyde and glyceraldehyde 3-phosphate are 96–97% hydrated at 25 °C, but it is the free aldehyde that is the substrate for glyceraldehyde-3-phosphate dehydrogenase. The equilibrium deuterium isotope effect for dehydration of an aldehyde hydrate is 1.37 for deuteration at C-1 (Lewis & Wolfenden, 1977). The equilibrium deuterium isotope effect for formation of a thiohemiacetal from a free aldehyde is 0.80 at C–1 (Lewis & Wolfenden, 1977), while that for transfer of the hydrogen from the thiohemiacetal to NAD can be calculated from the fractionation factors of an aldehyde hydrate and NADH (Cleland, 1980) and the above values as 1.05. In agreement with these estimates, the product of which is 1.15, the overall equilibrium deuterium isotope effect for the reaction was determined by direct comparison of K_{eq} values for unlabeled and 1-deuterated glyceraldehyde 3-phosphate as 1.145 ± 0.003.

The equilibrium ^{13}C isotope effects on the various steps of the glyceraldehyde-3-phosphate dehydrogenase reaction (at C-1 of the aldehyde substrate) were estimated at follows. To measure the isotope effect on aldehyde dehydration, a 10-mL solution of 370 mM D-glyceraldehyde was incubated at 2 °C for 3 s with sufficient GAPDH (2400 units) to completely oxidize the free aldehyde present in this time period. The reaction was then quenched with acid, and glycerate was isolated as usual. Since the rate constant for dehydration of the hydrate is 0.004 s^{-1} at 2 °C (Rendina et al., 1984) and and there is 37 times as much hydrate as free aldehyde at this temperature, as much as 44% of the original free aldehyde concentration could have been generated during this time by dehydration. The ^{13}C content of this additional material will depend on the ^{13}C isotope effect on dehydration, but in any case we estimate that the glyceric acid formed during the incubation period was ~55% from the original aldehyde and ~45% from aldehyde generated by dehydration.

The ^{13}C content of C-1 of the glyceric acid formed was determined by oxidation with periodate (see Materials and Methods) and compared with that of glycerate produced by total oxidation of a sample of D-glyceraldehyde with NAD, arsenate, and GAPDH. The resulting equilibrium ^{13}C isotope effect of 1.0028 ± 0.0009 for the dehydration is probably a maximum value, since the primary ^{13}C isotope effect on dehydration to form free aldehyde is probably greater than the equilibrium isotope effect of the reaction.

An attempt was made to determine the equilibrium ^{13}C isotope effect on thiohemiacetal formation by including sufficient mercaptoethanol (400 mM) in the reaction mixture to convert 51% of the glyceraldehyde to a thiohemiacetal. The free aldehyde present that was converted to glycerate in 3 s was now enriched in ^{13}C at C-1, rather than depleted, and a calculated value of 1.004 ± 0.001 was derived for the equilibrium ^{13}C isotope effect on thiohemiacetal formation from aldehyde and thiol. This experiment suffers from the same problem as the previous one, and thus the value is again only approximate. There seems little doubt, however, that the ^{13}C fractionation factor of C-1 in the thiohemiacetal is lower than that of the free aldehyde, which is in turn lower than that of the hydrate. Whether the overall difference is as large as 0.7% as these values indicate is questionable, however.

The equilibrium ^{13}C isotope effect for the oxidation of 5-thioglucose 6-phosphate to 5-thiogluconolactone 6-phosphate by NADP was measured by bringing the reaction catalyzed by glucose-6-phosphate dehydrogenase to equilibrium and allowing it to stand for 20 times longer to achieve isotopic equilibrium (30 units/mL glucose-6-phosphate dehydrogenase; the reaction goes at 0.6% the rate with glucose 6-phosphate but has essentially the same equilibrium constant). The total incubation time of 10 min at pH 5.9 was not sufficient for hydrolysis of an appreciable amount of the 5-thiogluconolactone 6-phosphate (0.1% per hour at this pH). The reaction was then stopped with acid, and, after readjustment to pH 7.5, the 5-thiogluconate 6-phosphate was oxidized with 6-phosphogluconate dehydrogenase (1 unit/mL; the rate is the same as with 6-phosphogluconate) to produce CO_2 from C-1 for isotope ratio mass spectrometric analysis. A sample of 5-thioglucose 6-phosphate was totally oxidized and analyzed similarly. From the data, the equilibrium ^{13}C isotope effect for the conversion from thiohemiacetal to thioester was calculated as 0.9941 ± 0.0004. This value should also apply to the oxidation of the thiohemiacetal of glyceraldehyde 3-phosphate or glyceraldehyde catalyzed by GAPDH.

The next step in the GAPDH-catalyzed reaction is the reaction of the thioester with phosphate to give 1,3-bisphosphoglycerate and a free cysteine. This reaction is similar to that catalyzed by phosphotransacetylase in which acetyl-CoA is converted to acetyl phosphate. This latter reaction (10 mL with 10 mM acetyl-CoA, 112 mM potassium phosphate, pH 7.0, 1 mM CoA, 15 mM acetyl phosphate, 10 units/mL phosphotransacetylase) was brought to equilibrium and incubated for 15 min (equilibrium was reached in <2 min). The reaction mixture was then cooled to 4 °C, and acetyl-CoA and acetyl phosphate were separated by ion-exchange chromatography on a 1.5 × 12.6 cm column of Dowex-1-Cl (elution

Table I: Equilibrium Isotope Effects

reaction	^{13}C	deuterium
aldehyde hydrate → aldehyde	1.0028 ± 0.0009	1.34[a]
aldehyde → thiohemiacetal	1.004 ± 0.001	0.80[a]
thiohemiacetal → thioester	0.9441 ± 0.0004	
thiohemiacetal → NADH		1.05[a,b]
thioester → acyl phosphate	0.9972 ● 0.0013	
overall reaction		
aldehyde hydrate → NADH		
exptl		1.145 ± 0.003
calc		1.15[c]
aldehyde hydrate → acyl phosphate		
exptl	0.9950 ± 0.0014	
calc	0.9982 ± 0.0019[c]	

[a] Lewis and Wolfenden (1977). [b] Cleland (1980). [c] Calculated from the product of values for the individual steps listed above.

with 1 mM phosphate buffer, pH 7, containing KCl was carried out in three stages: 30 mL of 90 mM KCl were used and the eluate was discarded; 40 mL of 150 mM KCl eluted the acetyl phosphate; and 50 mL of 400 mM KCl eluted the acetyl CoA). Acetyl phosphate and acetyl-CoA were each hydrolyzed with base to acetate. The sodium acetate samples were pyrolyzed on a vacuum line in the presence of NaOH at 400 °C to give CO_2 from the carboxyl group, and comparison of the ^{13}C contents allowed calculation of the equilibrium ^{13}C isotope effect for the conversion of acetyl-CoA to acetyl phosphate as 0.9972 ± 0.0013.

The GAPDH-catalyzed reaction was brought to equilibrium so that 5–6% of glyceraldehyde 3-phosphate was converted to bisphosphoglycerate. Equilibrium was reached in 5 s, and the total incubation time was 1.2 min. The enzyme was removed from the reaction mixture by ultrafiltration, and alkaline phosphatase was used to hydrolyze the bisphosphoglycerate. Glycerate was isolated by ion-exchange chromatography and degraded with periodate. Samples of glyceraldehyde 3-phosphate were also totally converted to 3-phosphoglycerate with arsenate. The equilibrium ^{13}C isotope effect for the reaction from the hydrate of glyceraldehyde 3-phosphate to bisphosphoglycerate was calculated as 0.9950 ± 0.0014.

The product of the equilibrium ^{13}C isotope effects corresponding to the four steps in the mechanism is 0.9982 ± 0.0019, which is in reasonable agreement with the measured overall value of 0.995. As noted above, however, there is considerable uncertainty in the values for dehydration and thiohemiacetal formation, and the fact that 0.9982 exceeds 0.995 by 0.3% presumably reflects this problem. In any case, it is clear that the equilibrium ^{13}C isotope effects are small, although in the expected directions. Thus, relative to free aldehyde the ^{13}C fractionation factors are 0.5% higher for an acyl phosphate and 0.2% higher for a thioester, while a hydrate is less than 0.3% higher and a thiohemiacetal up to 0.4% lower. Bonds to oxygen are clearly more stiffening than bonds to sulfur. Table I summarizes the equilibrium isotope effects discussed above.

Kinetic Isotope Effects. ^{13}C isotope effects were determined with either glyceraldehyde 3-phosphate or glyceraldehyde as substrates by following the change in ^{13}C content of C-1 of the substrate. A 3-phosphoglycerate kinase coupling system removed the 1-phosphate from the products to make the reaction irreversible, and the 3-phosphate of 3-phosphoglycerate was removed after the reaction was stopped by addition of alkaline phosphatase. The glycerate was then purified by ion-exchange chromatography and oxidized with periodate to give CO_2 from C-1. The initial ^{13}C content of C-1 was determined by substituting arsenate for phosphate so that the substrate was completely converted to product. The observed isotope effects are in Table II, as well as the calculated values corrected for the equilibrium ^{13}C isotope effect on aldehyde dehydration (that is, so the values reflect the isotope effect for reaction of the free aldehyde). Although this equilibrium isotope effect is somewhat uncertain (see above), the relative sizes of the kinetic isotope effects are certainly valid.

Deuterium isotope effects were determined by direct comparison of the kinetics of deuterated and unlabeled aldehyde substrates at $0.1K_m$ levels of phosphate. Under these conditions, equal isotope effects should be (and were) observed on the apparent V and V/K values,[3] which improves the precision of determining the isotope effects. With glyceraldehyde 3-phosphate, the isotope effect was 2.55 ± 0.12, while with glyceraldehyde it was 2.70 ± 0.09. After correction for the equilibrium deuterium isotope effect on aldehyde dehydration, the corrected values for the free aldehyde as substrate were 1.89 ± 0.09 for glyceraldehyde 3-phosphate and 2.00 ± 0.07 for glyceraldehyde. The α-secondary deuterium isotope effect resulting from deuterium substitution at C-4 of the nicotinamide ring of NAD was determined by varying the concentration of glyceraldehyde 3-phosphate with $0.1K_m$ phosphate and 2 mM NAD or NAD-4-*d*. The average of five determinations was 1.051 ± 0.023.

Discussion

Significant deuterium and ^{13}C isotope effects are seen on the GAPDH-catalyzed reaction with both glyceraldehyde 3-phosphate and glyceraldehyde as substrates. The ^{13}C isotope effect is considerably increased when the hydrogen involved in hydride transfer is deuterated and increased to some extent by α-secondary deuteration of NAD in the 4 position of the nicotinamide ring. These changes target hydride transfer as the major step responsible for the observed isotope effects and show that primary or secondary deuteration, by slowing this step, allows further expression of the ^{13}C isotope effect.

Hermes et al. (1982) developed equations (which assumed all isotope effects to be on the same step) for combining ^{13}C isotope effects (with unlabeled and primary and secondary deuterated substrates) with the primary and secondary deuterium isotope effects to solve for the intrinsic primary and secondary and ^{13}C isotope effects and the commitments. When the data from the present study were used with these equations, the intrinsic primary deuterium isotope effect was 4.95 ± 0.29, the α-secondary deuterium isotope effect was 1.43 ± 0.09, and the intrinsic ^{13}C isotope effect was 1.040 ± 0.003. The sum of forward and reverse commitments was 3.1 ± 0.3, but the distribution between them was not well determined (c_f = 1.1 ± 1.2; c_r = 2.0 ± 1.1). With glyceraldehyde as substrate, we have only ^{13}C isotope effects with primary deuterated or unlabeled substrates and the primary deuterium isotope effect, but substitution of these values into the equations of Hermes et al. (1982) gave 3.5 for the primary deuterium and 1.034–1.037 for the primary ^{13}C isotope effects, with the sum of commitments being 1.3–1.5.

The problem with these calculations is that more than one step is sensitive to primary ^{13}C and deuterium substitution. Formation of a thiohemiacetal involves primary ^{13}C and secondary deuterium isotope effects, with accompanying equilibrium isotope effects of ~1.0041 and 0.80 (see Results). The hydride transfer step involves primary ^{13}C and deuterium

[3] The apparent K_m for the aldehyde substrate when P_i is nonsaturating is its dissociation constant, which is not sensitive to deuterium substitution. Thus the apparent values of both V and V/K for the aldehyde show only the isotope effect on V.

isotope effects, with equilibrium isotope effects of 0.9941 and 1.05, and this is the only step that shows an α-secondary deuterium isotope effect (equilibrium isotope effect of 0.89; Cook et al., 1980). The reaction of the enzyme-bound thioester of 3-phosphoglycerate with phosphate to give 1,3-bisphosphoglycerate will show a primary ^{13}C isotope effect (equilibrium isotope effect of 0.9972) but is not sensitive to primary or secondary deuterium substitution.

The mechanism that describes the GAPDH-catalyzed reaction is

$$\text{E–NAD–GAP} \underset{k_2}{\overset{k_1[P_i]}{\rightleftharpoons}} \text{E–NAD–GAP–P}_i \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{E–NAD–THA–P}_i \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \text{E–NADH–TE–P}_i \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \text{E–NADH–BPG} \xrightarrow{k_9} \text{E–NADH + BPG} \quad (7)$$

where GAP is glyceraldehyde 3-phosphate, THA is the enzyme-bound thiohemiacetal of GAP, TE is the enzyme-bound thioester of 3-phosphoglycerate, and BPG is 1,3-bisphosphoglycerate. This mechanism assumes that the reaction is ordered, as found by Orsi and Cleland (1972), and that the chemistry does not occur until after all three substrates are present.[4] When phosphate concentration is held at $0.1K_m$ or less, the partition ratio of the E–NAD–GAP complex becomes unimportant, and thus the equations describing the isotope effects determined in the present work are

$$^{D}(V/K) = [^{D}K_{eq3}{}^{D}k_5 + (k_5/k_4)(^{D}k_3 + k_3/k_2) + {}^{D}K_{eq3}{}^{D}K_{eq5}(k_6/k_7)(1 + k_8/k_9)]/[1 + (k_5/k_4)(1 + k_3/k_2) + (k_6/k_7)(1 + k_8/k_9)] \quad (8)$$

$$^{\alpha\text{-}D}(V/K) = [^{\alpha\text{-}D}k_5 + (k_5/k_4)(1 + k_3/k_2) + {}^{\alpha\text{-}D}K_{eq}(k_6/k_7)(1 + k_8/k_9)]/[1 + (k_5/k_4)(1 + k_3/k_2) + (k_6/k_7)(1 + k_8/k_9)] \quad (9)$$

$$^{13}(V/K)_H = [^{13}K_{eq3}{}^{13}k_5 + (k_5/k_4)(^{13}k_3 + k_3/k_2) + {}^{13}K_{eq3}{}^{13}K_{eq5}(k_6/k_7)[^{13}k_7 + (k_8/k_9)^{13}K_{eq7}]]/[1 + (k_5/k_4)(1 + k_3/k_2) + (k_6/k_7)(1 + k_8/k_9)] \quad (10)$$

$$^{13}(V/K)_D = [^{13}K_{eq3}{}^{13}k_5 + (k_5/k_4)[^{D}k_3/(^{D}k_5{}^{D}K_{eq3})] \times [^{13}k_3 + k_3/(k_2{}^{D}k_3)] + {}^{13}K_{eq3}{}^{13}K_{eq5}(k_6/k_7)[^{D}K_{eq5}/{}^{D}k_5][^{13}k_7 + (k_8/k_9)^{13}K_{eq7}]]/[1 + (k_5/k_4)[^{D}k_3/(^{D}k_5{}^{D}K_{eq3})] \times [1 + k_3/(k_2{}^{D}k_3)] + (k_6/k_7)[^{D}K_{eq5}/{}^{D}k_5](1 + k_8/k_9)] \quad (11)$$

$$^{13}(V/K)_{\alpha\text{-}D} = [^{13}K_{eq3}{}^{13}k_5 + [k_5/(k_4{}^{\alpha\text{-}D}k_5)](^{13}k_3 + k_3/k_2) + {}^{13}K_{eq3}{}^{13}K_{eq5}(k_6/k_7)[^{\alpha\text{-}D}K_{eq5}/{}^{\alpha\text{-}D}k_5][^{13}k_7 + (k_8/k_9)^{13}K_{eq7}]]/[1 + [k_5/(k_4{}^{\alpha\text{-}D}k_5)] \times (1 + k_3/k_2) + (k_6/k_7)[^{\alpha\text{-}D}K_{eq5}/{}^{\alpha\text{-}D}k_5](1 + k_8/k_9)] \quad (12)$$

In this mechanism, $^{D}K_{eq3} = 0.80$, $^{D}K_{eq5} = 1.05$, $^{\alpha\text{-}D}K_{eq} = 0.89$, $^{13}K_{eq3} = 1.0041$, $^{13}K_{eq5} = 0.9941$, and $^{13}K_{eq7} = 0.9972$. There are, however, six unknown intrinsic isotope effects, plus the various partition ratios (k_3/k_2, k_5/k_4, k_7/k_6, k_9/k_8), with only five equations. One can solve for limits on key values, however, by assuming reasonable values for other parameters. We have assumed either that k_3/k_2 and k_8/k_9 were zero or unity and assumed values of $^{D}k_3$ between 0.80 and 0.99 (the reasonable range for this secondary isotope effect). $^{13}k_3$ and $^{13}k_7$ were then allowed to vary from 1.00 to 1.02 in all combinations, and the five equations were solved for $^{D}k_5$, $^{13}k_5$, $^{\alpha\text{-}D}k_5$, and k_5/k_4 and k_6/k_7. There was very little sensitivity to the assumed value of $^{D}k_3$, but, in order to obtain reasonable values for the isotope effects on k_5, $^{13}k_3$ and $^{13}k_7$ both had to be less than 1.01, and most likely 1.005 or less. The minimum values of $^{D}k_5$, $^{13}k_5$, and $^{\alpha\text{-}D}k_5$ were then 5.5, 1.034, and 1.36. These values differ only slightly from those derived earlier on the assumption of a single isotope-sensitive step.

[4] This assumption may not be completely valid, but the majority of the chemistry probably occurs only after all substrates add.

Table II: Kinetic ^{13}C Isotope Effects at pH 8

substrate[a]	observed $^{13}(V/K)$	corrected $^{13}(V/K)$[b]
GAP, NAD	1.0125 ± 0.0003	1.0097 ± 0.0003
GAP-1-*d*, NAD	1.0283 ± 0.0005	1.0254 ± 0.0005
GAP, NAD-4-*d*	1.0160 ± 0.0007	1.0132 ± 0.0007
GA, NAD	1.0179 ± 0.0006	1.0150 ± 0.0006
GA-1-*d*, NAD	1.0291 ± 0.0008	1.0262 ± 0.0008

[a] GAP, glyceraldehyde 3-phosphate. GA, glyceraldehyde. Phosphate concentration was $0.1K_m$ (0.4 mM) in partial conversion samples, while 10 mM arsenate was substituted in complete conversion samples. The values for the isotope effects are based on four determinations for the first two numbers and six for the other three. [b] Corrected for dehydration of the aldehyde, assuming an equilibrium isotope effect of 1.0028. These values thus refer to the free aldehyde as substrate.

These are reasonable values for the intrinsic isotope effects on hydride transfer, but the large normal α-secondary deuterium isotope effect (the equilibrium isotope effect is inverse, 0.89) shows that the motion of primary and secondary hydrogens is tightly coupled in the transition state and involves tunneling (Cook et al., 1981; Huskey & Schowen, 1983; Saunders, 1984). This is, in fact, the largest α-secondary deuterium isotope effect on such a hydride transfer observed to date, the previous highest value being that seen with formate dehydrogenase [1.23, Hermes et al. (1984)].

When the isotope effects with glyceraldehyde 3-phosphate and glyceraldehyde as substrates are compared, it is clear that there are somewhat smaller commitments with the latter so that the isotope effects are more fully expressed. However, there still are commitments present, since the ^{13}C isotope effect is increased with glyceraldehyde 1-*d*, and our analysis on the assumption of a single isotope-sensitive step suggests the commitments are about half those seen with glyceraldehyde 3-phosphate. Glyceraldehyde is a slow substrate with a V_{max} 5% that of glyceraldehyde 3-phosphate, and catalysis is slowed by an even greater ratio, since V_{max} is limited by NADH release with glyceraldehyde 3-phosphate, but presumably by the chemistry with glyceraldehyde (Orsi & Cleland, 1972). We do not know which partition ratio is still finite with glyceraldehyde, but it is most likely that for the thiohemiacetal intermediate (k_5/k_4 in mechanism 7) or possibly for the thioester (k_6/k_7). The partition ratio of the E–NAD–GA–P_i complex (k_3/k_2) is unlikely to have a very large value, since the absence of the orienting phosphate group in the 3 position of glyceraldehyde probably makes thiohemiacetal formation a slow step. These data illustrate the fact that in a reaction where multistep chemistry is involved, a very slow substrate often is slow getting started but shows nearly normal partitioning of intermediates during the chemical reaction. Glyceraldehyde is possibly slow in forming a thiohemiacetal because of the lack of a phosphate group at C-3 to anchor it in place.

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Mechanism of Serpin Action: Evidence That C1 Inhibitor Functions as a Suicide Substrate†

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Received April 18, 1991; Revised Manuscript Received June 27, 1991

ABSTRACT: Serpins form a family of structurally related proteins, many of which function in plasma as inhibitors of serine proteases involved in inflammation, blood coagulation, fibrinolysis, and complement activation. To further characterize the mechanism by which serpins inhibit their target enzymes, we have studied the effect of temperature on the reaction of C1 inhibitor and the serine protease plasma kallikrein. At both 38 and 4 °C, C1 inhibitor (M_r 105000) is cleaved by α-kallikrein (M_r 85000 and 88000) at position P_1 (Arg^{444}) of the reactive center, a reaction that leads to the formation of a covalent bimolecular enzyme–serpin complex (M_r 195000) and cleaved but uncomplexed serpin (M_r 95000). Between 38 and 4 °C, the product distribution is temperature-dependent, with more cleaved C1 inhibitor (M_r 95000) formed at lower temperatures and correspondingly less M_r 195000 complex. Studies employing intrinsic tryptophan fluorescence and ^1H NMR spectroscopy show that this behavior is not caused by temperature-dependent conformational changes of kallikrein or C1 inhibitor. C1 inhibitor also behaves in this manner with the light chain of kallikrein and, to a lesser extent, with plasmin and $C\bar{1}s$. These data are best explained by a branched reaction pathway, identical with the scheme describing the mechanism of action of suicide substrates. This scheme involves the formation of an enzyme–inhibitor intermediate, which can be stabilized into a covalent complex and/or dissociate into free enzyme and cleaved inhibitor, depending on the reaction conditions.

C1 inhibitor, the missing or dysfunctional protein in hereditary angioedema (Donaldson & Evans, 1963), is a member of the serpin superfamily of protease inhibitors, which also includes α_1-antitrypsin, antithrombin III, and the plasminogen activator inhibitors (Huber & Carrell, 1989; Schapira & Patston, 1991). C1 inhibitor is the only inhibitor in serum for the $C\bar{1}r$ and $C\bar{1}s$ subcomponents of the first component of complement (Sim et al., 1979). C1 inhibitor is also the predominant inhibitor of the blood coagulation cascade enzymes plasma kallikrein and activated factor XII, although some of the inhibitory capacity of normal plasma against kallikrein is mediated by α_2-macroglobulin (Schapira et al., 1982; van der Graaf et al., 1983a; de Agostini et al., 1984; Pixley et al., 1985), antithrombin III (Olson & Choay, 1989), and activated protein C inhibitor (Meijers et al., 1988). Using assays specific for kallikrein–C1 inhibitor and kallikrein–α_2-macroglobulin complexes, Harpel et al. (1985) showed that, in normal plasma activated by kaolin, temperature has a dramatic effect on the distribution of this serine protease between the two inhibitors. At 37 °C, 67% of kallikrein is bound to C1 inhibitor and 33% to α_2-macroglobulin; at 4 °C, 85% is bound to α_2-macroglobulin and 15% to C1 inhibitor (Harpel et al., 1985). Earlier evidence that C1 inhibitor is inefficient at low temperature was provided by studies where normal plasma was supplemented with radioiodinated prekallikrein, activated with kaolin, and the distribution of ^{125}I-kallikrein between various inhibitors was evaluated by SDS–PAGE and autoradiography (van der Graaf et al., 1983a), and by investigations on kallikrein-dependent cold-promoted activation of factor VII (van Royen et al., 1978). However, the above-referenced studies did not address the question of why C1 inhibitor is inefficient at low temperature. The relative inefficiency of C1 inhibitor in the cold was also noticed by Cameron et al. (1989).

In the present paper, we describe a study in a purified system of the effect of temperature on the reactivity of C1 inhibitor with plasma kallikrein. Although C1 inhibitor was cleaved by kallikrein at an identical site at 38 and 4 °C, it functioned more efficiently as an inhibitor at 38 °C than at 4 °C. However, even at 38 °C, there was significant proteolysis of C1 inhibitor without enzyme inhibition. Since C1 inhibitor displayed this behavior with other serine proteases, it is a

† This work was supported in part by National Institutes of Health Grant HL-40875 (to M.S.).
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0006-2960/91/0430-8876$02.50/0