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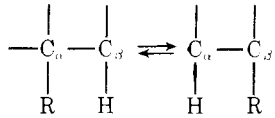
Mechanism of Action of Adenosylcobalamin: Hydrogen Transfer in the Inactivation of Diol Dehydratase by Glycerol†

William W. Bachovchin,‡ Kevin W. Moore,‡ and John H. Richards*

ABSTRACT: We have investigated the kinetic characteristics of the inactivation of the adenosylcobalamin-dependent enzyme propanediol dehydratase by glycerol, (*RS*)-1,1-dideuterioglycerol, (*R*)-1,1-dideuterioglycerol, and perdeuterioglycerol in the presence of 1,2-propanediol and 1,1-dideuterio-1,2-propanediol. The results imply that hydrogen (or deuterium) attached to C-1 of 1,2-propanediol participates in the inactivation process and contributes to the expression of a kinetic isotope effect on the rate of inactivation. The mechanism for this inactivation must involve the cofactor as an intermediate hydrogen carrier, presumably in the form of 5′-deoxyadenosine. Moreover, a mechanism involving a rate-determining transfer of hydrogen from an intermediate containing three equivalent hydrogens quantitatively accounts for all of the results. When diol dehydratase holoenzyme is inactivated by [1-^{3}H]glycerol, 5′-deoxyadenosine which is enriched in tritium by a factor of 2.1 over that in glycerol can be isolated from the reaction mixture.

A number of enzymes require adenosylcobalamin (AdoCbl[1]) as cofactor and catalyze reactions of the general type:



where R can be alkyl, amino, hydroxyl, or carbonyl (Hogenkamp, 1968; Babior, 1975a,b). Propanediol dehydratase is an AboCbl-dependent enzyme which catalyzes the rearrangement of both (*R*)- and (*S*)-1,2-propanediol to propionaldehyde and of ethylene glycol to acetaldehyde.

Until recently (Toraya et al., 1976; Bachovchin et al., 1977), diol dehydratase was thought to be inactive toward glycerol. This behavior distinguished propanediol dehydratase from another very similar AboCbl-dependent enzyme, glycerol dehydratase, which catalyzes the rearrangement of glycerol to β-hydroxypropionaldehyde as well as acting upon 1,2-propanediol and ethylene glycol. In fact, propanediol dehydratase holoenzyme does catalyze the rearrangement of glycerol to β-hydroxypropionaldehyde. However, glycerol also causes rapid and irreversible inactivation of the enzyme, thus explaining why catalytic activity toward glycerol was not detected previously (Lee and Abeles, 1963). Both the inactivation and catalysis reactions exhibit large primary deuterium isotope effects (k_H/k_D = 14 and 8, respectively); thus, hydrogen transfer is an important rate-contributing step in each case. Experiments with various isotopically substituted glycerols demonstrated that two diastereomeric combinations are possible between the enzyme and glycerol; the enzyme–substrate complexes were designated "EG_R" and "EG_S". When glycerol is bound to enzyme in the "EG_R" complex, a hydrogen is abstracted from the pro-R carbon and the catalytic rate actually exceeds that observed with 1,2-propanediol as substrate. When glycerol is bound in the "EG_S" complex, hydrogen is abstracted

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[1] Abbreviations used are: AdoCbl, adenosylcobalamin; NADH, reduced nicotinamide adenine dinucleotide.

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from the *pro-S* carbon and inactivation, rather than catalysis, follows as the dominant event (Bachovchin et al., 1977).

A number of other modified substrates (such as isobutylene glycol, thioglycerol, and 3-chloro-1,2-propanediol) also function as irreversible inactivators of diol dehydratase and display a number of similarities to glycerol in their interaction with diol dehydratase holoenzyme. For example, in all cases 1,2-propanediol acts as a purely competitive inhibitor of inactivation. With glycerol as inactivator this behavior was extensively studied and shown to occur over a wide range of both glycerol and 1,2-propanediol concentrations (Bachovchin et al., 1977).

Irreversible inactivation has been observed with ethylene glycol and diol dehydratase and with glycerol and glycerol dehydratase, although the inactivation rates are considerably slower than that of glycerol inactivation of diol dehydratase (Lee and Abeles, 1963; Poznanskaya et al., 1972). Thus, irreversible inactivation by substrate appears to be a rather common phenomenon associated with these enzymes and a study of its mechanism may advance our understanding of the normal catalytic mechanism.

Accordingly, we undertook a detailed study of the mechanism of inactivation. In this paper, we report the kinetics of glycerol inactivation of diol dehydratase holoenzyme in the presence of 1,2-propanediol and 1,1-dideuterio-1,2-propanediol and discuss their bearing on inactivation and catalysis.

Experimental Section

Enzyme Preparations. Propanediol dehydratase [(*RS*)-1,2-propanediol hydro-lyase; EC 4.2.1.28] was obtained from *Klebsiella pneumoniae* (ATCC 8724) by a procedure similar to that of Lee and Abeles (1963). Fraction E-8 with a specific activity of 25–50 was used for all determinations. 1,2-Propanediol-free enzyme was prepared as previously reported (Frey et al., 1967).

Adenosylcobalamin. Adenosylcobalamin (AdoCbl) was purchased from Sigma Chemical Co.

5′-Deoxyadenosine. 5′-Deoxyadenosine was prepared by the intermediate synthesis of *N*-acetyl-5′-iodo-2′,3′-isopropylideneadenosine by a previously reported method (Jahn, 1965; McCarthy et al., 1968). 5′-Deoxyadenosine was also prepared directly from 5′-deoxy-5′-iodoadenosine (Aldrich Chemical Co.) by catalytic hydrogenation using previously described conditions (McCarthy et al., 1968). The materials obtained by both methods were identical.

Substrates. 1,2-Propanediol, 1,1-dideuterio-1,2-propanediol, glycerol, perdeuterioglycerol, (*RS*)-1,1-dideuterioglycerol, (*R*)-1,1-dideuterioglycerol, and [1-^{14}C]glycerol were obtained as described by Bachovchin et al. (1977).

[*1*-^{3}*H*]*Glycerol* was purchased from Amersham.

Determination of Inactivation Rates. Inactivation rates were determined using the coupled enzyme assay (Bachovchin et al., 1977). Reaction mixtures, in general, consisted of: yeast alcohol dehydrogenase, 15 units; NADH, 0.2 mM; diol dehydratase, 0.05–0.5 unit; bovine serum albumin, 0.12 mg; potassium phosphate buffer (pH 8.0), 30 mM; adenosylcobalamin, 0.02 mM; and the desired amount of 1,2-propanediol and/or inactivator. The total volume was 2.50 mL. The reaction mixture was incubated at 37 °C with stirring. The inactivation reaction was started by the addition of AdoCbl and stopped by the addition of 50 μL of a 6 M 1,2-propanediol solution. Enzyme activity (measured by the rate of production of propionaldehyde) was determined at a minimum of four different inactivation times; the slope of the corresponding semilog plot was taken as the observed inactivation rate constant ($k_{i,obsd}$) for the given concentration of inactivator and 1,2-propanediol.

TABLE I: Chromatography of 5′-Deoxyadenosine.

Method/solvent	R_f of 5′-dAdo
Paper	
n-BuOH–H_2O (43:7)	0.34
n-BuOH–HOAc–H_2O (12:3:5)	0.60
n-BuOH–HOAc–H_2O (4:1:5)	0.77
1 M NH_4OAc–95% EtOH–H_2O (9:20:1)	0.75
Silica gel	
Water-saturated *n*-BuOH + 1% NH_3	0.35
sec-BuOH–H_2O–NH_4OH (50:18:7)	0.27

Inactivation by [*1*-^{3}*H*]*Glycerol.* A typical solution was composed of the following: apoenzyme, 40 units; [1-^{3}H]glycerol, 0.49 μCi/μmol (0.142 M); K_2HPO_4 buffer, 10 mM; AdoCbl, 0.025 mM; total volume, 0.50 mL, 37 °C. In control experiments, the apoenzyme solution was either boiled for 2 min or inactivated by the addition of 0.01 mL of 6.4 mM CNCbl prior to the addition of AdoCbl. The holoenzyme–[1-^{3}H]glycerol solution was allowed to react for 1 h at 37 °C in the dark. 5′-Deoxyadenosine (0.15 mL of a 2 mg/mL solution) was added as carrier, and 0.05 mL of 6.4 mM CNCbl solution was added to visualize subsequent chromatography. The solution was immersed in boiling H_2O for 2 min and then applied to a 2.6 × 16 cm column of Bio-Gel P-2 (200–400 mesh) and eluted in 4-mL fractions with 0.015 N NH_3/0.02% NaN_3 buffer in the dark. The nucleoside-containing fractions were pooled, acidified to pH 3.0 with 2 N HCl, and applied to a 1 × 9 cm AG50W-X2 (200–400 mesh) column previously equilibrated with 10^{-3} M HCl. The column was washed with 100 mL of H_2O to remove radioactive glycerol. The nucleoside was eluted with 0.1 N NH_3, lyophilized, dissolved in a small amount of methanol, and applied to strips of Whatman 3MM paper or to silica gel plates. Paper chromatograms were developed by the descending method in the solvent systems given in Table I; silica gel plates were developed by the ascending method.

The nucleoside was visualized by its ultraviolet extinction and eluted from the chromatogram with methanol. The methanol was removed under vacuum and the residue was taken up in 1 mL of H_2O and added to 10 mL of scintisol complete or PCS–xylene (2:1) and assayed for radioactivity. Standardization was accomplished by using [α-^{3}H]- and [α-^{14}C]toluene of known specific activities.

Results

Kinetics of Inactivation by Perdeuterioglycerol. The rate of inactivation of diol dehydratase holoenzyme by perdeuterioglycerol (gly-d_5) was determined as a function of the concentration of gly-d_5 in the absence of 1,2-propanediol under anaerobic conditions at 37 °C. As with glycerol, the rates of inactivation are always first order in active holoenzyme. (The concentration of gly-d_5 was assumed to be essentially constant throughout the inactivation reaction, as only a negligible fraction of the initial gly-d_5 was converted to β-hydroxypropionaldehyde.) Accordingly, we can apply simple Michaelis–Menten equations that have been suitably modified to account for a decreasing amount of catalytically active holoenzyme (Bachovchin et al., 1977). Thus,

$$[E_a] = [E_a]_0 e^{-k_{i,obsd}t} \quad (1)$$

where $[E_a]_0$ is the concentration of active enzyme present initially, $[E_a]$ is the concentration of enzyme, both free and

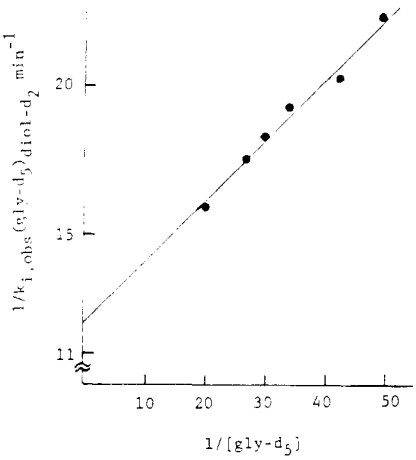


FIGURE 1: Double-reciprocal plot for inactivation by gly-d_5 in the presence of 1.16×10^{-4} M diol-d_2; slope = 0.22; intercept = 11.8.

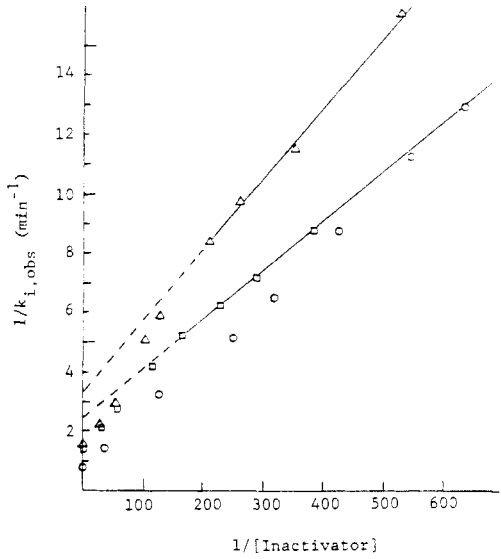


FIGURE 2: Plot of the reciprocal of the observed inactivation rate constant in the presence of 1.08×10^{-4} M diol-d_2 vs. the reciprocal of inactivator concentration: (○) glycerol; (□) (*R*)-gly-d_2; (△) (*RS*)-gly-d_2. The values depicted at infinite inactivator concentration represent values obtained experimentally at 0.04 M inactivator in the absence of 1,2-propanediol.

with inactivator bound in a Michaelis–Menten complex, which remains active at any time t, $k_{i,obsd}$ is the observed first-order rate constant for inactivation and is given by:

$$k_{i,obsd} = \frac{k_i}{1 + \frac{K_G}{[G]}} \quad (2)$$

where k_i is the true first-order rate constant for inactivation and K_G is the dissociation constant for the enzyme–glycerol complex

$$K_G = \frac{[E_a][G]}{[E_aG]}$$

A plot of $1/k_{obsd}$ vs. $1/[gly\text{-}d_5]$ is linear; from the slope and intercept values, K_G and k_i were obtained. The value of K_G determined for gly-d_5 (3.9×10^{-3} M) differs somewhat from that of glycerol (1.6×10^{-3} M) obtained by the same method (Bachovchin et al., 1977). The value of k_i obtained from the y intercept is 0.085 min^{-1}, indicating an isotope effect of 15 on the rate constant for inactivation. This represents reasonable agreement with the value of 14 previously obtained by comparison of the observed rate constants for gly-d_5 and gly at 0.04 M in the absence of 1,2-propanediol; the discrepancy can, nevertheless, be quantitatively explained by the different contributions made by oxygen inactivation to $k_{i,obsd}$ due to the different K_G values.

Kinetics of Glycerol Inactivation. Competition with (RS)-1,1-Dideuterio-1,2-propanediol. The rate of inactivation of holoenzyme in the presence of a fixed concentration of (*RS*)-1,1-dideuterio-1,2-propanediol (diol-d_2) was determined as a function of concentration of inactivator for the following inactivators: glycerol (gly), (*RS*)-1,1-dideuterioglycerol [(*RS*)-gly-d_2], (*R*)-1,1-dideuterioglycerol [(*R*)-gly-d_2], and perdeuterioglycerol (gly-d_5). A fixed concentration of diol-d_2 of 1.08×10^{-4} M was chosen for two reasons: (1) This concentration is sufficiently large to validate the assumption that $\Delta[S] \sim 0$ over the time interval during which we determined the inactivation rates. (2) This concentration is sufficiently small to allow large rates of inactivation to occur at reasonable concentrations of inactivators.

Figures 1 and 2 illustrate the results. With gly-d_5 as inactivator (Figure 1), the double-reciprocal plot is linear over the entire range of inactivator concentration. Moreover, in this case, the y intercept is nearly identical to that obtained for the substrate-free case. Thus, eq 3, previously derived to describe the inactivation kinetics of gly in the presence of undeuterated 1,2-propanediol (Bachovchin et al., 1977), should again apply.

$$k_{i,obsd}(gly)_{diol} = \frac{k_i(gly)}{\left(1 + \frac{K_G(gly)}{[gly]}\right)\left(1 + \frac{[diol]}{K_i(diol)}\right)} \quad (3)$$

The slope of the double-reciprocal plot with gly-d_5 as inactivator should be given by

$$\text{slope} = \left(\frac{K_G(\text{gly-}d_5)}{k_i(\text{gly-}d_5)}\right)\left(1 + \frac{[\text{diol-}d_2]}{K_i(\text{diol-}d_2)}\right)$$

Since values of K_G(gly-d_5) and k_i(gly-d_5) are available, a value of K_i(diol-d_2) of 3.0×10^{-5} M can be calculated. This is the same as the value of K_i for undeuterated diol previously obtained in an analogous manner with glycerol as inactivator (Bachovchin et al., 1977) and demonstrates that there is no deuterium isotope effect on K_i for the competitive inhibition by propanediol of the inactivation of holoenzyme by glycerol.

In contrast to data obtained for gly-d_5, double-reciprocal plots for (*R*)- and (*RS*)-gly-d_2 appear to become linear only at low inactivator concentrations (Figure 2). Data for gly appear linear as plotted in Figure 2; however, they actually exhibit behavior similar to that of (*R*)- and (*RS*)-gly-d_2: downward curvature at high inactivator concentration and linearity at low inactivator concentration. (Figure 3 better illustrates this behavior, which is discussed later in detail.) In the linear regions, data for gly and (*R*)-gly-d_2 define the same line, whereas data for (*RS*)-gly-d_2 give a line with different slope and y intercept. Slopes of the linear regions should be (eq 3):

$$\text{slope} = \left(\frac{K_G(G)}{k_i(G)}\right)\left(1 + \frac{[\text{diol-}d_2]}{K_i(\text{diol-}d_2)}\right)$$

where G is gly, (*R*)- or (*RS*)-gly-d_2. Assuming the y intercept obtained by extrapolation of the linear region to infinite inactivator concentrations to be the reciprocal of the respective k_i, one may use the value of K_i for diol-d_2 to calculate a value of K_G for each inactivator. In each case, this value is 1.6×10^{-3} M, the same as that previously determined for gly independent

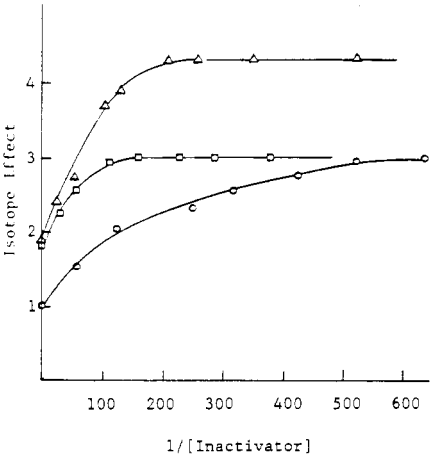


FIGURE 3: Plot of the isotope effect on the observed inactivation rate constant vs. the reciprocal of inactivator concentration in the presence of 1.08×10^{-4} M diol-d_2: (○) gly; (□) (*R*)-gly-d_2; (△) (*RS*)-gly-d_2. The isotope effects were calculated from the corresponding experimentally determined values of $k_{i,obsd}$ as described under Results.

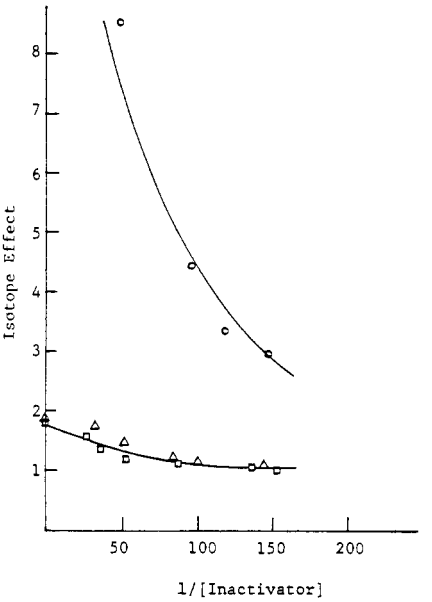


FIGURE 4: Plot of the isotope effect on the observed rate of inactivation vs. the reciprocal of inactivator concentration in the presence of 1.4×10^{-3} M diol: (○) gly-d_5; (△) (*RS*)-gly-d_2; (□) (*R*)-gly-d_2. The isotope effects were calculated from the corresponding experimentally determined values of $k_{i,obsd}$ as described under Results.

of the presence of 1,2-propanediol (Bachovchin et al., 1977). Thus, although gly-d_5 exhibits a K_G somewhat different from that of gly, neither (*R*)- nor (*RS*)-gly-d_2 manifest a deuterium isotope effect on K_G.

A plot of the observed isotope effect vs. 1/[inactivator] (Figure 3) more informatively illustrates the variation of $k_{i,obsd}$. The isotope effects were calculated by comparing $k_{i,obsd}$ measured at each concentration of a given inactivator in the presence of diol-d_2 to the $k_{i,obsd}$ obtained for the corresponding concentrations of undeuterated glycerol and propanediol. For example, the isotope effect on inactivation at a given concentration of (*R*)-gly-d_2 would be defined as:

$$\left(\frac{k_H}{k_D}\right)_i = \frac{k_{i,obsd}(gly)_{diol}}{k_{i,obsd}((R)\text{-gly-}d_2)_{diol\text{-}d_2}} \quad (4)$$

$k_{i,obsd}((R)\text{-gly-}d_2)_{diol\text{-}d_2}$ is the experimentally determined "observed" inactivation rate constant at the particular concentration of (*R*)-gly-d_2 in the presence of the fixed concentration of diol-d_2 and $k_{i,obsd}(gly)_{diol}$ is the corresponding observed rate constant for gly in the presence of undeuterated diol. Use of eq 3 and the fact that [gly] = [(*R*)-gly-d_2] and [diol] = [diol-d_2] reduce eq 4 to:

$$\left(\frac{k_H}{k_D}\right)_i = \frac{k_i(gly)}{k_i((R)\text{-gly-}d_2)}$$

The data in Figure 3 show that, for all three inactivators, one approaches a limiting maximum isotope effect at low inactivator concentration. For (*RS*)-gly-d_2, this value is 4.3; for gly and (*R*)-gly-d_2 the value is 3.0.

Kinetics of Glycerol Inactivation. Competition with (RS)-1,2-Propanediol. The rate of inactivation in the presence of a fixed concentration of undeuterated diol was determined as a function of inactivator concentration for the following inactivators: (*RS*)-gly-d_2, (*R*)-gly-d_2, and gly-d_5. The analogous experiment with undeuterated glycerol as inactivator has previously been reported (Bachovchin et al., 1977); the kinetics are described by eq 3 with $k_1 = 1.3$ min^{-1} and $K_G = 1.6 \times 10^{-3}$ M. Figure 4 illustrates the results as a plot of the expressed isotope effect on inactivation vs. the reciprocal of inactivator concentration. Isotope effects were calculated as described in the previous section.

Isolation and Identification of 5′-Deoxyadenosine. When [1-^{3}H]glycerol inactivates diol dehydratase holoenzyme, tritium is transferred to the nucleoside moiety of AdoCbl. This nucleoside chromatographs with authentic 5′-deoxyadenosine on silica gel and Whatman 3MM paper in a number of solvent systems. The amount of tritium in the 5′-deoxyadenosine isolated after each reaction is directly proportional to the amount of holoenzyme inactivated; control experiments using [1-^{14}C]glycerol or heat-inactivated apoenzyme yielded no radioactivity associated with 5′-deoxyadenosine. A least-squares fit of ^{3}H dpm in 5′-deoxyadenosine as a function of the amount of enzyme indicates that 5′-deoxyadenosine is enriched in tritium by a factor of approximately 2.1 over substrate, on a per mole basis. This calculation assumes an essentially quantitative liberation of 5′-deoxyadenosine from holoenzyme.

A control experiment in which the inactivated holoenzyme was not denatured prior to chromatography on Bio-Gel P-2 showed that the nucleoside remains bound to the protein and does not exchange with unlabeled 5′-deoxyadenosine in solution.

Discussion

The kinetics of inactivation when both inactivator and inhibitor are "homogeneous" with respect to transferrable hydrogens (i.e., undeuterated glycerol with undeuterated propanediol, or gly-d_5 with diol-d_2) obey eq 3, with appropriate constant values for K_G, K_i, and k_i, for all concentrations of inactivator at the fixed "inhibitor" concentration. This is not true, however, for the isotopically "mixed" cases [i.e., undeuterated glycerol, (*RS*)-gly-d_2 and (*R*)-gly-d_2 with diol-d_2 or gly-d_5, (*RS*)-gly-d_2 and (*R*)-gly-d_2 with undeuterated diol) as illustrated by the double-reciprocal plots (for example, Figure 2). Possible sources for this divergent behavior may be variation of K_G, K_i, and/or k_i with concentration of inactivator.

One can construct a mechanism whereby k_i may vary by drawing upon the currently accepted catalytic mechanism and the supporting experimental data. Catalysis by diol dehydratase holoenzyme and other AdoCbl-dependent enzymes involves two hydrogen-transfer steps: (1) from C-1 of substrate to C-5′ of adenosylcobalamin and (2) from C-5′ of adenosyl-

TABLE II: Comparison of the Limiting Values of the Inactivation Rate Constants and Resulting Isotope Effects.

	Substrate free		1,2-Propanediol-d_2		1,2-Propanediol	
	k_i (min^{-1})	$(k_H/k_D)_i$	k_i (min^{-1}),[a] 1.08×10^{-4} M diol-d_2	k_H/k_D	k_i,[a] 1.4×10^{-3} M diol	$(k_H/k_D)_i$
Glycerol	1.3	1.0	0.43	3.0	1.30	1.0
(*R*)-Glycerol-d_2	0.72	1.8	0.43	3.0	1.30	1.0
(*RS*)-Glycerol-d_2	0.68	1.9	0.30	4.3	<1.30	>1.0
Glycerol-d_5	0.085	15.0	0.087	15.0	≈0.90	≈1.4

[a] The k_i values listed in the competition with substrate experiments are the constant k_i's fitting eq 3 at low inactivator concentrations.

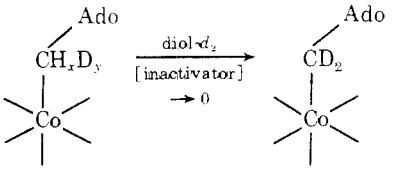
cobalamin to C-2 of product (Abeles, 1971). Evidence for the formation of 5′-deoxyadenosine as an intermediate and evidence for the equivalence of the C-5′ hydrogens, although not conclusive, are quite extensive (Babior, 1975b; Frey et al., 1967; Miller and Richards, 1969; Eagar et al., 1972). Thus, both intramolecular and intermolecular transfers of hydrogen are possible (Abeles and Zagalak, 1966). Moreover, the isotope effect on the second hydrogen-transfer step (cofactor → product) considerably exceeds that for the first hydrogen-transfer step; thus, deuterium (or tritium) accumulates at the 5′-carbon of cofactor during catalysis. Indeed, enrichment in tritium of enzyme-bound AdoCbl by as much as 20-fold over that in substrate has been observed during catalysis (Essenberg et al., 1971). Thus, a mechanism exists for the "inhibitor" of inactivation to contribute a hydrogen (or deuterium) to the inactivation pathway. Furthermore, the isotopic composition of the 5′ carbon of cofactor, and thus the isotope effect expressed on hydrogen transfer from cofactor, should vary with inactivator concentration. For the "homogeneous" cases, no such variation should occur, since only hydrogen or only deuterium is involved in the reaction. Thus, the divergent behavior observed between the homogeneous and mixed cases can be explained in terms of the variation of k_i and requires only that a rate-contributing or rate-limiting step for inactivation occur at some point along the pathway after the initial abstraction of hydrogen from C-1 of glycerol. This step could be hydrogen abstraction either from C-5′ of the resulting 5′-deoxyadenosine intermediate or from C-1 of glycerol after equilibration between this hydrogen and those attached to C-5′ of AdoCbl. However, hydrogen exchange between cofactor and unreacted substrate could not be demonstrated in the diol dehydratase (Frey et al., 1967) and ethanolamine deaminase (Carty et al., 1971) reactions. In the case of diol dehydratase, ethylene glycol, a substrate which also inactivates holoenzyme, was used. Thus, equilibration of hydrogen between AdoCbl and reversibly bound substrate is unlikely. If such exchange occurs after an irreversible step such as reaction 1 above, no distinction can be drawn between these two suggested mechanisms; both require formation of an intermediate containing three equivalent hydrogens.

Alternatively, changes in K_G or K_i (eq 3) with concentration of inactivator may be considered as an explanation for the double-reciprocal plots in the mixed cases. This explanation, however, appears unlikely. The inhibitor Michaelis constant, K_i, for 1,2-propanediol is unaffected by deuterium substitution. While gly-d_5 does exhibit an isotope effect on K_G, neither (*RS*)-gly-d_2 nor (*R*)-gly-d_2 does. (The reason for the isotope effect of gly-d_5 on K_G is presently obscure.) Moreover, the effects observed when undeuterated diol protects against gly-d_5 are too large to be explained in terms of effects on K_G or K_i. Thus, an explanation which relies on the effect of deuterium substitution in inhibitor on the rate constant for inactivation remains the most tenable.

We shall, therefore, argue that not only does transfer of hydrogen from cofactor contribute to the observed inactivation rate constant, thereby implicating the presence of an intermediate such as 5′-deoxyadenosine in the inactivation pathway, but that transfer of hydrogen from this intermediate is the rate-determining step in the inactivation process. All our kinetic data accord with this hypothesis; in particular, the limiting value of the isotope effects observed at low inactivator concentrations (Figures 3 and 4) can be quantitatively explained by this hypothesis.

We shall consider first the "mixed" cases in which diol-d_2 functions as the inhibitor of inactivation. At very high concentrations of inactivator, diol-d_2 should be effectively excluded from the holoenzyme, and the rate of inactivation should, and does, extrapolate to that observed for inactivation in the absence of diol inhibitor (Figures 2 and 3; Table II lists the rates of inactivation and the corresponding isotope effects observed for each inactivator in the absence of propanediol). As the concentration of inactivator decreases, an increasing proportion of holoenzyme interacts with diol-d_2, and enzyme-bound AdoCbl becomes increasingly rich in deuterium. Thus, the inactivation rate constant reflects an increasing isotope effect (Figure 3).

Figure 3 also illustrates that, as inactivator concentration decreases, the observed isotope effect reaches a limiting maximum value for each inactivator, a behavior consistent with our hypothesis. Considering the much larger isotope effect on the second hydrogen-transfer step (cofactor → product) one might expect that, as the concentration of inactivator relative to inhibitor is decreased, a point should be reached where essentially all transferrable hydrogens at C-5′ of enzyme-bound AdoCbl have been replaced with deuterium.

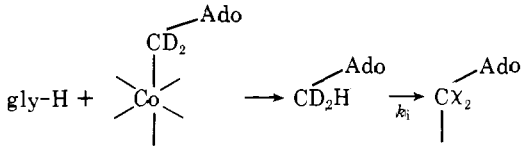


(x and y represent the isotopic composition that would characterize the cofactor.) Thus, when the cofactor is saturated with deuterium, no further increase in the isotope effect with decreasing inactivator concentration would be possible, and a maximum value for the isotope effect for each particular inactivator would be reached as demonstrated experimentally in Figure 3.

The inactivator concentration at which this maximum, limiting value is expressed differs for each inactivator. With (*R*)-gly-d_2, this "saturation" effect occurs at a relatively high concentration of (*R*)-gly-d_2; with (*RS*)-gly-d_2, a somewhat lower concentration is required; with undeuterated glycerol, this saturation effect is observed only at very low concentrations of inactivator. This order likewise accords with the pro-

posed hypothesis. We have previously shown that glycerol not only inactivates holoenzyme, it can also serve as a substrate (a reaction involving predominantly the *pro-R* end of the glycerol molecule) (Bachovchin et al., 1977). (*R*)-gly-d_2 contains only deuterium on the *pro-R* carbon; thus, catalysis involving this glycerol as substrate should more readily enrich enzyme-bound AdoCbl with deuterium than would catalysis involving (*RS*)-gly-d_2, which contains both deuterium and hydrogen in equal amounts on the *pro-R* carbon. Undeuterated glycerol acting as substrate should actually retard the saturation of enzyme-bound AdoCbl with deuterium. Thus, the observed order follows from the hypothesis and the ability of glycerol to act both as substrate and inactivator.

The maximum limiting values of the isotope effects are 3.0 both for undeuterated glycerol and for (*R*)-gly-d_2, while for (*RS*)-gly-d_2 the experimental value is 4.3 (Figure 3). These values can be quantitatively explained by the hypothesis. At very low concentrations, glycerol in the presence of diol-d_2 finds enzyme-bound AdoCbl saturated with deuterium.

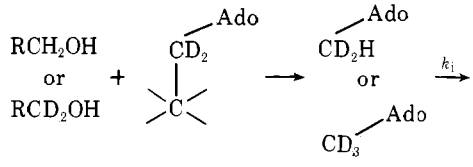


where X = D or H

Since glycerol contains only hydrogen, the resulting 5′-deoxyadenosine must be $-CD_2H$. Using 15 as the value for the primary isotope effect in transfer away from 5′-deoxyadenosine and assuming that the three hydrogens have a statistically equal chance to be removed in this rate-determining step, one calculates that the isotope effect should be:

$$\left(\frac{k_H}{k_D}\right)_i = [(2/3)(1/15) + (1/3)(1)]^{-1} = 2.7$$

This agrees reasonably well with the experimental value of 3.0. With (*R*)-gly-d_2, again only hydrogen can be transferred to AdoCbl during inactivation, since inactivation occurs predominantly from the *pro-S* end of glycerol (Bachovchin et al., 1977); thus, the calculated value is the same as that for undeuterated glycerol which agrees with the experimental value. However, with (*RS*)-gly-d_2 either deuterium or hydrogen can be transferred, so that two isotopically substituted types of 5′-deoxyadenosine are possible.



Assuming an equal contribution of both types of 5′-deoxyadenosine to the rate and the statistical equivalence of the three hydrogens at C-5′, one calculates that the isotope effect in this case should be:

$$\left(\frac{k_H}{k_D}\right)_i = \left[\frac{0.5}{[(2/3)(1/15) + (1/3)(1)]^{-1}} + \frac{0.5}{15}\right]^{-1} = 4.5$$

which agrees well with the experimental value.

In contrast, the isotope effects calculated for a mechanism involving an intermediate with two equivalent hydrogens would be 1.9 for gly and (*R*)-gly-d_2 and 3.3 for (*RS*)-gly-d_2. Thus, the experimental data favor a mechanism involving the intermediacy of a species with three equivalent hydrogens.

Further verification of this hypothesis comes from evaluation of the Michaelis constants for inactivation, K_G, and for inhibition, K_i, by quantitative analysis of these data for "mixed" inhibition. With k_i constant, eq 3 should again apply, and, in fact, the double-reciprocal plots of these points are linear with slopes and intercepts indicating the appropriate new values of k_i while indicating no effect on either K_G or K_i for each inactivator (Figure 2). Thus, the proposed hypothesis accounts for all of the features of the experimental results presented in Figures 2 and 3.

We now consider the corresponding "mixed" cases in which undeuterated propanediol is the inhibitor of inactivation. In these cases, the deuterium content of enzyme-bound AdoCbl should decrease with decreasing concentration of inactivator and the observed isotope effect should also decrease.

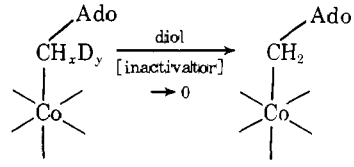


Figure 4 illustrates the agreement between this expectation and the experimental results. This effect is more clearly illustrated with gly-d_5 as inactivator; at high concentrations of gly-d_5, the isotope effect approaches 15, but with decreasing concentrations of gly-d_5 the observed isotope effect decreases dramatically.

Again, as one lowers the concentration of inactivator relative to diol inhibitor, enzyme-bound AdoCbl should become saturated with the isotope present in the diol, which in this case is hydrogen rather than deuterium. Thus, one anticipates a limiting *minimum* value for the isotope effect. The results in Figure 4 suggest that such a limit is in fact approached for each inactivator. However, in practice, experimental data in this region of "constant k_i" cannot be obtained for many reasons.

Nevertheless, limiting minimum values for the isotope effects expected for each inactivator can be calculated on the basis of the above hypothesis; these values agree rather well with those approached experimentally (Figure 4). For example, (*R*)-gly-d_2 with only hydrogen at the *pro-S* carbon can generate only an Ad–CH_3 intermediate; the calculated isotope effect for inactivation is thus 1.0. The calculated value for (*RS*)-gly-d_2 is only slightly larger (1.2). With both these inactivators, the experimental value appears to approach 1 as the limiting minimum value. Gly-d_5 can, however, only contribute deuterium and the intermediate in the limiting case must be $AdoCH_2D$. The isotope effect calculated for this intermediate is 1.4, considerably smaller than the value of 15 observed at very large gly-d_5 concentrations. Thus, this case presents the theory with a challenging test. In fact, the experimental data do indicate such a large change and seem to approach a limiting minimum somewhat greater than 1 (Figure 4).

Summary and Conclusions

The results of this work indicate the occurrence of an intermediate such as 5′-deoxyadenosine along the inactivation pathway and, furthermore, that transfer of hydrogen from such an intermediate containing three equivalent hydrogens is the rate-determining step in the inactivation process. This hypothesis can account quantitatively for the limiting values of the isotope effects on k_i observed at low inactivator concentrations in the presence of fixed concentrations of diol and diol-d_2, which function as competitive inhibitors of inactivation. The hypothesis also accounts for the variation of k_i with

the concentration of inactivator in the cases which are isotopically "mixed" and for the invariance of k_i in the isotopically "homogeneous" cases. Accordingly, we conclude that the mechanism of inactivation, like that of catalysis, involves as the first step abstraction of hydrogen from C-1 of glycerol and transfer of this hydrogen to the 5′-carbon of cofactor. The three hydrogens thereby attached to C-5′ of deoxyadenosine become equivalent and removal of one of them is involved in the rate-determining step for inactivation. Whether this transfer from 5′-deoxyadenosine involves readdition of hydrogen to the glycerol skeleton or transfer to some other group or species associated with the enzyme or coenzyme remains unclear, as does the nature of the inactivated holoenzyme complex. The identification of the nucleoside in the inactivated complex as 5′-deoxyadenosine suggests that the 5′-deoxyadenosyl moiety which obtains after the second hydrogen-transfer step is capable of acquiring a third hydrogen, possibly either from substrate or a nearby amino acid residue, since intact AdoCbl cannot be recovered following glycerol inactivation (Bachovchin et al., 1977).

A 5′-deoxyadenosine moiety containing three equivalent hydrogens has long been considered as an intermediate in catalysis. The significance of these results for the mechanism of catalysis is that they establish kinetically the feasibility of such an intermediate in the mechanism of glycerol inactivation, a mechanism which is very similar to and may in fact be identical with the mechanism of catalysis. The major unique feature of inactivation could be that enzyme-bound "product" formed in the normal way from glycerol bound in an "EG_S" fashion effects irreversible inactivation of the holoenzyme complex.

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Studies on the Microsomal Mixed Function Oxidase System: Redox Properties of Detergent-Solubilized NADPH-Cytochrome P-450 Reductase†

Takashi Iyanagi,* F. Koichi Anan, Yoshio Imai,‡ and Howard S. Mason§

ABSTRACT: Hepatic microsomal NADPH-cytochrome P-450 reductase was solubilized from rabbit liver microsomes in the presence of detergents and purified to homogeneity by column chromatography. The purified reductase had a molecular weight of 78 000 and contained 1 mol each of FAD and FMN per mol of enzyme. On reduction with NADPH in the presence of molecular oxygen, an O_2-stable semiquinone containing one flavin free radical per two flavins was formed, in agreement with previous work on purified trypsin-solubilized reductase. The reduction of oxidized enzyme by NADPH, and autoxidation of NADPH-reduced enzyme by air, proceeded by both one-electron equivalent and two-electron equivalent mechanisms. The reductase reduced cytochrome P-450 (from phenobarbital-treated rabbits) and cytochrome P-448 (from 3-methylcholanthrene-treated rabbits). The rate of reduction of cytochrome P-450 increased in the presence of a substrate, benzphetamine, but that of cytochrome P-448 did not.

Hepatic NADPH-cytochrome P-450 reductase (EC 1.6.2.4), the flavoprotein component of a liver microsomal mixed-function oxidase, contains 1 mol each of FAD and FMN per mol of enzyme. The two flavins have different properties, e.g., oxidation–reduction characteristics (Iyanagi & Mason, 1973; Iyanagi et al., 1974). An active mixed-function oxidase system can be reconstituted from microsomal fractions containing the NADPH-cytochrome P-450 reductase, cytochrome P-450, and phosphatidylcholine (Lu & Coon, 1968). These components have been purified in several laboratories. The purified detergent-solubilized NADPH-cytochrome P-450 reductase, which also contains one molecule each of FAD and FMN, can reduce cytochrome P-450 directly in a reconstituted

† From the Division of Biochemistry, Tsukuba University School of Medicine, Sakura, Niihari, Ibaraki 300-31, Japan. *Received October 31, 1977.*

‡ Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan.

§ Department of Biochemistry, University of Oregon Health Sciences Center, Portland, Oregon 97201.

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