Suicide Inactivation of Dioldehydrase by 2-Chloroacetaldehyde: Formation of the '*cis*-Ethanesemidione' Radical, and the Role of a Monovalent Cation

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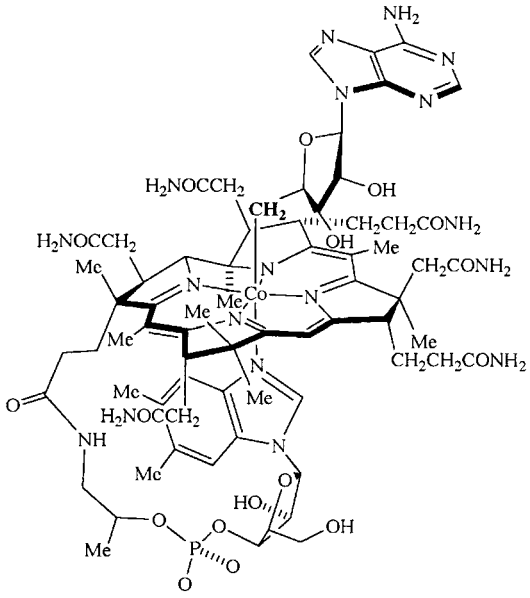
This paper is dedicated with appreciation and gratitude to Professor *Duilio Arigoni*, a great scientist and a great friend

Dioldehydrase is an adenosylcobalamin-dependent enzyme that catalyzes the dehydration of (*R*)- or (*S*)-propane-1,2-diol to propanal. The reaction proceeds by a radical mechanism initiated by the homolytic scission of the covalent Co–C(5′) bond in the coenzyme to form cob(II)alamin and the 5-deoxyadenosyl radical as transient intermediates. Dioldehydrase is subject to 'suicide inactivation' by substrate/product analogs. Inactivation by 2-chloroacetaldehyde converts the inactivator into the '*cis*-ethanesemidione' radical. A mechanism for this process includes reaction of chloroacetaldehyde in the reverse of the normal catalytic process to a rearranged radical that eliminates HCl. K^+ and other monovalent cations of similar size, including Tl^+, are required for dioldehydrase activity and for suicide inactivation by glycolaldehyde or 2-chloroacetaldehyde. A K^+ ion is bound to propane-1,2-diol in dioldehydrase. Both EPR and pulsed-EPR experiments show that the magnetic nuclei of thallous ions ($^{203}Tl^+$, $^{205}Tl^+$) do not interact with the unpaired electron in the *cis*-ethanesemidione radical at the active site of dioldehydrase. Pulsed-EPR experiments implicate a ^{14}NH group, possibly of His^{143}, interacting with the radical at the active site.

Introduction. – Dioldehydrase (EC 4.2.1.28) catalyzes the elimination of H_2O from (*R*)- or (*S*)-propane-1,2-diol to produce propanal. From a chemical standpoint, a polar reaction mechanism would not seem to be feasible under physiological conditions of temperature and pH. Presumably for this reason, the evolved enzyme employs the radical-initiating vitamin B_{12} coenzyme, adenosylcobalamin (**1**). Adenosylcobalamin is a source of the 5′-deoxyadenosyl radical in enzymatic reactions. The action of adenosylcobalamin and the 5′-deoxyadenosyl radical in the reaction of dioldehydrase follows the course outlined in *Scheme 1* [1a].

The Co–C bond of **1** is weak (30 kcal mol^{-1}) [2] and undergoes reversible homolytic scission at the active site of dioldehydrase, leading to the formation of cob(II)alamin and the 5′-deoxyadenosyl radical. The H-transfer pathway shown in *Scheme 1* has been traced by tritium and deuterium labeling of the substrate, and observation of the label in both the product and intermediate 5′-deoxyadenosine [3]. After (*R*)-propane-1,2-diol binds in *step 1* to the complex of dioldehydrase and adenosylcobalamin, the 5′-deoxyadenosyl radical (Ado–$CH_2^{\bullet}$) abstracts $H_{pro\text{-}R}$ from C(1) in *step 2* to produce 5′-deoxyadenosine and the substrate-related radical (*R*)-propane-1,2-diol-1-yl. Rearrangement of this radical in *step 3* leads to the product-related radical propane-1,1-diol-2-yl, which abstracts a H-atom from the Me group of



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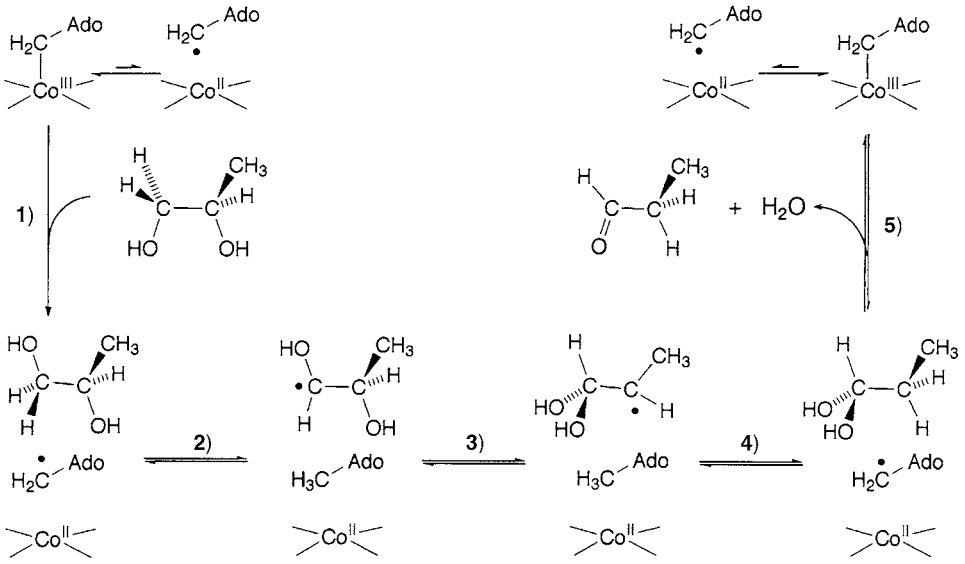
5′-deoxyadenosine in *step 4* to form propanal hydrate, regenerating the 5′-deoxyadenosyl radical. Dissociation of propanal hydrate from the enzyme is finally accompanied by dehydration to propanal [4a]. All steps are stereospecific, as indicated in *Scheme 1*, including the H abstraction from C(1) [4b], the migration of OH from C(2) to C(1) [4b], and the dehydration of the enzyme-bound propanal hydrate [4a]. H Transfer from C(1) to C(2) proceeds with inversion of configuration at C(2) in the reactions of both substrates [4b]. (*S*)-Propane-1,2-diol is nearly as good a substrate as its enantiomer, but reacts stereospecifically in the opposite way with respect to both H abstraction and dehydration to propanal [4].

Co–C Bond cleavage is not detectable by spectroscopic methods in the resting complex of dioldehydrase and adenosylcobalamin (**1**) [5]. Although adenosylcobalamin itself does not react with O-containing species, it is sensitive when bound to dioldehydrase [5], suggesting that binding forces between the enzyme and adenosylcobalamin in the complex weaken the Co–C bond. In the absence of the substrate, the enzyme-coenzyme complex reacts with O_2 to produce cob(III)alamin and products derived from the nucleoside moiety [5b]. The sensitivity to O_2 and the products of Co–C(5′) bond cleavage indicate the presence of a small equilibrium concentration of cob(II)alamin and the 5′-deoxyadenosyl radical at the active site. Upon addition of propane-1,2-diol to the complex of dioldehydrase and adenosylcobalamin, cleavage of the Co–C bond can be observed spectrophotometrically by the appearance of the cob(II)alamin spectrum, which is characterized by an absorption maximum at 477 nm [5a]. Upon transformation of all of the substrate into propanal, the spectrum returns to that of adenosylcobalamin. Thus, cob(II)alamin is an intermediate in this transformation.

Scheme 1. *The Role of Adenosylcobalamin* (**1**) *in the Reaction of Dioldehydrase.* The Co–C bond of **1** undergoes reversible homolytic scission at the active site of dioldehydrase to form cob(II)alamin and the 5′-deoxyadenosyl radical, which initiates the dehydration of propane-1,2-diol. The reaction mechanism is shown in five steps, beginning with the binding of the substrate (*step 1*). Several of the steps shown are, no doubt, composites of two or more processes. For example, the rearrangement of the substrate-related radical intermediate to the product related radical (*step 3*) is likely to proceed *via* two steps and an intermediate; and the release of the product (propanal) (*step 5*) is likely to involve at least two discrete steps. Cob(II)alamin and the substrate-related radical have been detected as intermediates by VIS spectrophotometry and EPR, and 5′-deoxyadenosine has been identified by chemical methods. The pathway of H transfer has been traced by labeling studies.



Electron-paramagnetic-resonance (EPR) spectroscopy reveals the presence of a substrate-based radical intermediate upon mixing the complex of dioldehydrase and adenosylcobalamin with propane-1,2-diol [6]. The EPR spectrum indicates the presence of this radical in the steady state, as well as cob(II)alamin, and both EPR signals disappear upon completion of the reaction. Kinetic studies have corroborated that the formation and decomposition of both the radical and cob(II)alamin are sufficiently fast to make them kinetically potential intermediates. Recent findings have suggested that the radical observed transiently is the product of H abstraction from propane-1,2-diol according to *Scheme 1* [1b].

Dioldehydrase is subject to 'suicide inactivation' in reactions with substrate analogs. Inactivation by glycolaldehyde (2-hydroxyacetaldehyde) produces an inactive complex consisting of the enzyme, cob(II)alamin, 5′-deoxyadenosine, and a C_2 product derived from glycolaldehyde [5a]. Inactivation by [2-^{3}H]glycolaldehyde leads to the incorporation of tritium into 5′-deoxyadenosine [5b]. All of the inactivation products remain tightly bound to dioldehydrase and are released upon denaturation of the enzyme.

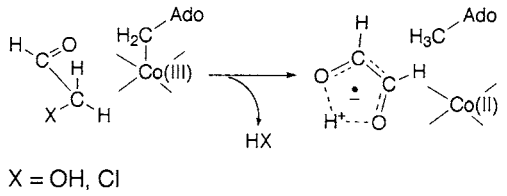
5′-Deoxyadenosine made its first appearance as a biochemical entity in this reaction in 1967. However, the C_2 product derived from glycolaldehyde was only recently identified as the '*cis*-ethanesemidione' radical[1]) [7a].

In the present paper, we revisit the reported suicide inactivation of dioldehydrase by 2-chloroacetaldehyde [8]. We also examine the question of whether the monovalent cation required for enzymatic activity and for inactivation by glycolaldehyde is engaged in an interaction with the product of suicide inactivation, the *cis*-ethanesemidione radical, at the active site.

Results. – *Suicide Inactivation of Dioldehydrase.* As in the inactivation of dioldehydrase by glycolaldehyde, 2-chloroacetaldehyde reacts with the complex of dioldehydrase and adenosylcobalamin (**1**) to cleave the Co–C(5′) bond, producing cob(II)alamin and 5′-deoxyadenosine [8]. The process leads to the concomitant transformation of 2-chloroacetaldehyde into a free radical tightly bound to dioldehydrase, presumably at the active site, as indicated by the EPR signal of the inactive complex. Inactivation by [^{13}C]chloroacetaldehyde leads to a broadened EPR signal, and reaction of [^{2}H]chloroacetaldehyde leads to a narrowed EPR signal, proving that the unpaired electron of the organic radical resides on the carbon skeleton of 2-chloroacetaldehyde [8].

The EPR spectrum of the radical generated in the inactivation of dioldehydrase by 2-chloroacetaldehyde turns out to be identical to that of the radical produced in the suicide inactivation by glycolaldehyde, as shown in *Fig. 1*. Inasmuch as the structure of the *cis*-ethanesemidione radical is rigorously established by the study of glycolaldehyde inactivation [7a], the identical spectrum for the product of 2-chloroacetaldehyde inactivation, as well as the identical responses of the spectra to ^{2}H- and ^{13}C-isotopic substitutions [8], show that this radical is the end product in *both* reactions. Inactivation by both compounds, therefore, leads to the same inactivated complex, consisting of dioldehydrase, cob(II)alamin, 5′-deoxyadenosine, and the *cis*-ethanesemidione radical. The chemical transformation occurring at the active site upon suicide inactivation by either compound can be formulated as in *Scheme 2*.

Scheme 2



X = OH, Cl

The Role of the Monovalent Cation. The catalytic activity of dioldehydrase depends on the presence of K^+ or another monovalent cation, preferentially one with an ionic radius similar to that of K^+. Suicide inactivation by glycolaldehyde also depends on the presence of K^+ [5]. Moreover, the published structures of dioldehydrase with propane-

[1]) Resonance structures of the 1-hydroxy-2-oxoethyl radical.

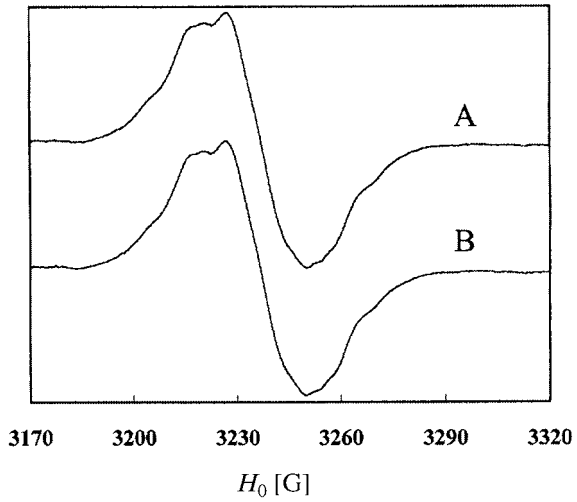


Fig. 1. *Detection of the* cis*-ethanesemidione radical as the product of suicide inactivation of dioldehydrase by 2-chloroacetaldehyde.* Shown are the EPR spectra of matched samples of dioldehydrase inactivated by glycolaldehyde (A) or chloroacetaldehyde (B). Spectra were obtained at 5-mW microwave power at 7 K and at a microwave frequency of *ca.* 9.1 GHz.

1,2-diol bound to the enzyme show the substrate to be coordinated with a K^+ ion [1b]. The interaction seems to represent solvation of the K^+ by the OH groups of propane-1,2-diol. This raises the question of whether K^+ is either coordinated or solvated by the *cis*-ethanesemidione radical within the suicide-inactivation complex. The magnetic properties of the radical can be exploited to address this question.

If the monovalent cation and the *cis*-ethanesemidione radical are coordinated, then an activating cation with nuclear spin should introduce the possibility of a magnetic interaction between the unpaired electron and the nucleus of the cation. Thallous ions (Tl^+), with nearly the same ionic radius as K^+, have the magnetically active nucleii $^{203,205}Tl$ ($I = 1/2$) and do activate dioldehydrase. The spectral changes shown in *Fig. 2* suggest that either K^+ or Tl^+ potentiate the suicide inactivation of dioldehydrase by 2-chloroacetaldehyde. Addition of 2-chloroacetaldehyde to the complex of dioldehydrase and adenosylcobalamin has no effect on the absorption spectrum in the absence of an activating monovalent cation. However, when either K^+ or Tl^+ are present, the addition of 2-chloroacetaldehyde quickly leads to the transformation of adenosylcobalamin into cob(II)alamin. Therefore, suicide inactivation by 2-chloroacetaldehyde requires the presence of a monovalent metal cation, as does inactivation by glycolaldehyde [5].

Electron-nuclear hyperfine coupling between the *cis*-ethanesemidione radical and Tl^+ as the activator would split the EPR signal of the radical. A very strong nuclear hyperfine coupling can be expected if Tl^+ were to replace the exchangeable proton in the *cis*-ethanesemidione radical. This chelate structure, though, cannot represent the radical at the active site of dioldehydrase because the radical retains the exchangeable proton [7a]. However, a weaker interaction with nonbonding electrons by means of

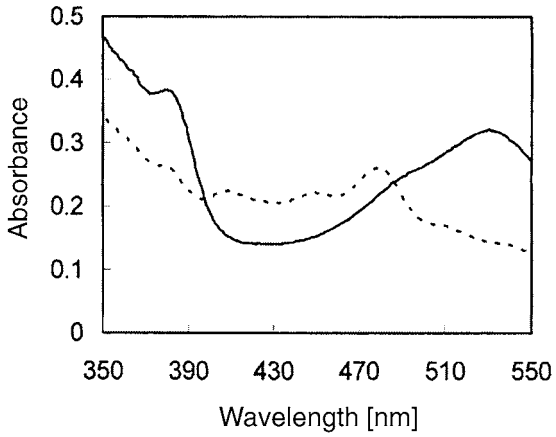


Fig. 2. *UV/VIS Spectra of matched samples of dioldehydrase* (**1**), *and glycolaldehyde* (7.5 mM) *without metal cation* (—) *or in the presence of* K^+ (25 mM) (– – – –). The two spectra correspond to adenosylcobalamin in the absence of, and to cob(II)alamin in the presence of K^+, resp. Identical results were obtained with 2-chloroacetaldehyde in place of glycolaldehyde.

solvation by propane-1,2-diol or H_2O, should result in a detectable splitting in the EPR spectrum [7b,c].

In *Fig. 3*,*a*, the EPR spectra of the inactivation complexes composed of dioldehydrase, cob(II)alamin, *cis*-(D_3)ethanesemidione, and K^+ or Tl^+ do not reveal any magnetic interactions between the radical and Tl^+. The complexes were made of dioldehydrase inactivated by addition of (D_3)glycolaldehyde in D_2O to make use of the line-narrowing effect of deuterium, and to minimize the magnitude of internal electron-nuclear hyperfine splitting, which is why the spectra are narrowed relative to those given in *Fig. 1*. This approach maximizes the chance to observe a magnetic interaction with Tl^+. Despite these measures, the spectrum obtained for the sample with Tl^+ is nearly identical to that obtained for K^+. The very slight differences in lineshape are smaller than the differences observed between the spectra recorded with K^+ and Na^+ (*Fig. 3*,*b*). Such differences represent secondary structural effects mediated through the protein to the active site and are attributed to small chemical differences in the binding of different monovalent ions to the enzyme. The absence of any magnetic effect of Tl^+ on the EPR properties suggests that *cis*-(D_3)ethanesemidione is not coordinated to Tl^+ in the active site.

Pulsed-EPR Analysis. A longer-range magnetic interaction of the thallium nuclear spin with the unpaired electron of *cis*-ethanesemidione would be revealed by ENDOR (electron-nuclear double resonance) spectroscopy. However, a careful analysis of dioldehydrase samples inactivated by glycolaldehyde in the presence of Tl^+ failed to reveal the presence of a Tl-ENDOR signal. Such a signal is expected when Tl^+ resides near (within a few Å) the unpaired electron. But neither EPR nor Tl-ENDOR spectroscopy showed any sign of either a direct or long-range contact between the *cis*-ethanesemidione radical and Tl^+ at the active site.

In ESEEM (electron-spin-echo envelope modulation) [9], a series of short, intense microwave pulses is applied to the sample. The sample then emits a transient response

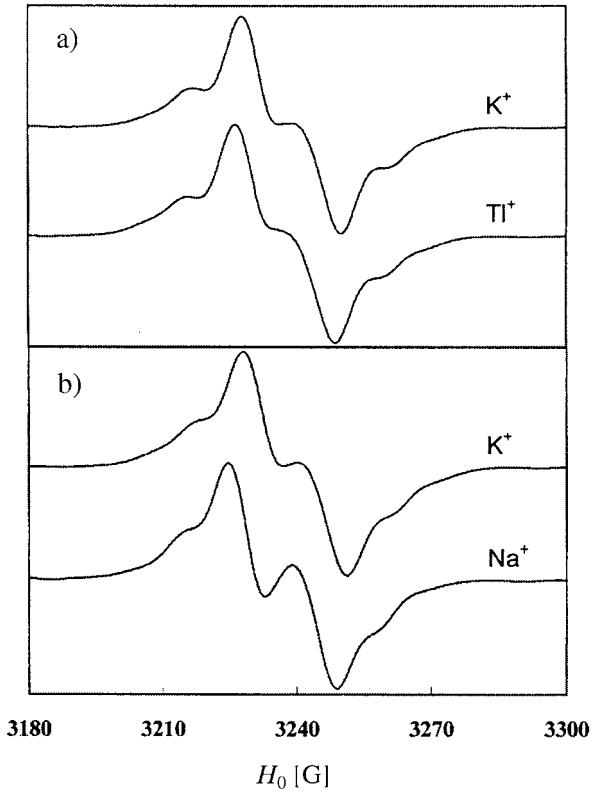


Fig. 3. *EPR Spectra of the* cis*-ethanesemidione radical in dioldehydrase complexes with K^+ or Tl^+*. EPR Spectra of the radical region for samples containing initially dioldehydrase and adenosylcobalamin (**1**) in D_2O after inactivation with [$^2H_3$]glycolaldehyde. Labels indicate that monovalent metal cations were present. a) *Matched samples inactivated in the presence of AcOK or AcOTl* (5 mM each). b) *Matched samples inactivated in the presence of AcOK or AcONa* (0.2 M each). Spectra were obtained at 5-mW microwave power, 77 K, microwave frequency: *ca.* 9.1 GHz.

called electron-spin echo. The intensity of the response decays as the delay between two adjacent pulses is increased. If a nearby magnetic nucleus is coupled to the unpaired electron, then the decay can be nonmonotonic, instead of exhibiting sinusoidal modulations of various periodicities. *Fourier* transformation (FT) of the modulations produces a spectrum of nuclear-spin transitions, to which only those nuclei within a few Å of the unpaired electron will generally make a detectable contribution.

In *Fig. 4*, *a* the three-pulse (stimulated echo) [10] ESEEM spectra of dioldehydrase inactivated by glycolaldehyde in the presence of either K^+ (blue) or Tl^+ (red) are shown. The spin-echo modulations are quite deep and contain a complex combination of periods, but the two traces are very similar. FT yielded nuclear-frequency spectra that were virtually identical in the 0.5–2.0 MHz range (*Fig. 4*, *b*), ruling out interactions with either metal ion as a source of the modulations. However, intense peaks appeared

at *ca.* 0.62, 0.92, and 1.57 MHz. Notably, the sum of the two lowest frequencies was 1.54 MHz, virtually the same as the highest frequency. This is characteristic of a weakly-coupled ^{14}N-nucleus in which, for one quantum state of the electron spin, the external magnetic field is cancelled by the magnetic field arising from the isotropic hyperfine interaction. In such a case, the ESEEM spectrum yields a pure set of nuclear electric quadrupole resonance frequencies, labeled ν_0, ν_-, and ν_+, respectively. The relationship between the observed ESEEM frequencies, thus, allowed us to assign them to ^{14}N, particularly in view of the lack of effect of changing the monovalent cation. Further, the values of these frequencies can be compared with those from NQR (nuclear-quadrupole-resonance) measurements of free amino acids and similar compounds. Considering that, based on the X-ray crystal structure, the most-likely candidates are the imidazole ring of His^{143} and the side chain carboxamido group of Gln^{296}, there are three possible catalytic groups: two histidines and one glutamine. The best fit was to the NH N-atom of the histidine ring, with NQR frequencies of 0.66, 0.75, and 1.42 MHz [11].

Discussion. – *Inactivation by* cis-*Ethanesemidione.* The discovery of *cis*-ethanesemidione as the product of suicide inactivation by either glycolaldehyde or 2-chloroacetaldehyde rationalizes the physicochemical basis for inactivation. The radical is symmetrical, and the unpaired electron is highly delocalized, so that it is too stable to abstract a H-atom from 5′-deoxyadenosine at an appreciable rate, thereby stopping the reaction in a quasi-intermediate state. The reaction cannot proceed in either the forward or reverse direction, both of which require abstraction of a H-atom from the Me group of 5′-deoxyadenosine. Binding interactions between dioldehydrase and all components within the quasi-intermediate state, as well as sequestration of the radical within the active site and out of contact with O_2 and reducing agents in the solvent, greatly inhibit the occurrence of side reactions that would quench the radical.

Mechanism of cis-*Ethanesemidione Formation.* The discovery of the *cis*-ethanesemidione radical as a product of suicide inactivation by glycolaldehyde did not lead to an obvious mechanism for radical formation, apart from the fact that it required H abstraction from glycolaldehyde by the 5′-deoxyadenosyl radical. The question remained whether glycolaldehyde hydrate reacts with dioldehydrase as an analog of propane-1,2-diol, or whether glycolaldehyde reacts by reversal of the overall mechanism (*Scheme 1*). Three mechanisms for suicide inactivation by glycolaldehyde have been put forward, two of which involve reaction of glycolaldehyde hydrate as an analog of propane-1,2-diol, in which an OH group of the hydrate replaced the Me group of propane-1,2-diol in the binding site. The third mechanism involves the simple abstraction of a H-atom from C(2) of glycolaldehyde proper [7a].

Our result that 2-chloroacetaldehyde produces the same radical, *cis*-ethanesemidione, as in the above studies cannot be rationalized by any of the mechanisms put forward. Reaction of 2-chloroacetaldehyde hydrate as a substrate analog, with Cl in place of one OH group, is highly unlikely, given that neither 1- nor 2-chloropropanol either react with or inhibit dioldehydrase at concentrations greater than 1M (data not shown). The inability of 2-chloroacetaldehyde hydrate to react as an analog of propane-1,2-diol rules out two of the mechanisms considered for the reaction of glycolaldehyde [7a]. And the reaction of 2-chloroacetaldehyde cannot be so simple as

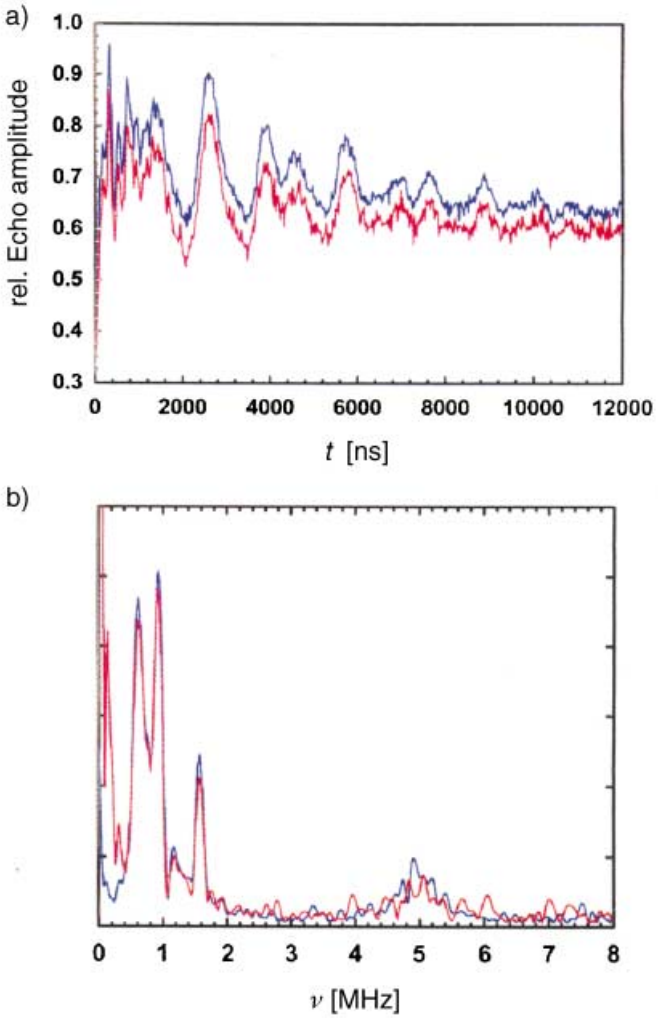
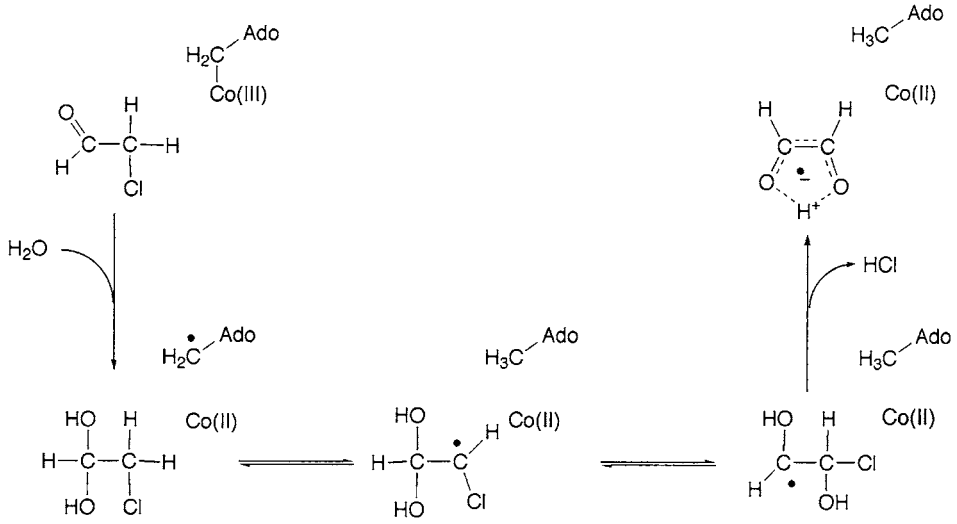


Fig. 4. *ESEEM Spectra of dioldehydrase inactivated by glycolaldehyde in the presence of* K^+ *or* Tl^+. a) *Stimulated-echo ESEEM spectra of samples prepared in the presence of either* K^+ (blue) *or* Tl^+ (red). b) *FT Nuclear-frequency spectra* (colors as in *a*). The intense transitions are assigned to ^{14}N interactions with a neighboring amino acid (see text).

postulated in the third mechanism for the glycolaldehyde reaction, which, in the case of 2-chloroacetaldehyde, would be H-atom abstraction by the 5′-deoxyadenosyl radical from C(2) of the aldehyde. Such a process could not lead to the *cis*-ethanesemidione radical. This radical must result from a process that involves, in addition to H-atom abstraction from C(2), the elimination of Cl^- and the incorporation of an O-atom at C(2).

A simple mechanism for suicide inactivation by 2-chloroacetaldehyde is outlined in *Scheme 3*. Its reaction as a product analog by reversal of the mechanism proposed in

Scheme 3. *A Mechanism for the Suicide Inactivation of Dioldehydrase by 2-Chloroacetaldehyde*. 2-chloroacetaldehyde undergoes reaction as the free aldehyde, following the reverse of steps in *Scheme 1*, including reverse migration of an OH group in the hydrate, until a substrate-related radical is produced, which suffers elimination of HCl to form the *cis*-ethanesemidione radical.



Scheme 1 leads to a radical species that can form the *cis*-ethanesemidione radical by elimination of HCl from the same C-atom. It is known that propanal, the product of the reaction of propane-1,2-diol, readily removes tritium from [5′-^{3}H]adenosylcobalamin [3c], showing that the reverse mechanism is possible (see *Scheme 1*). Analogous reactions of 2-chloroacetaldehyde would lead to *cis*-ethanesemidione formation and inactivation.

One cannot conclude that glycolaldehyde reacts in the same way as 2-chloroacetaldehyde. A simpler mechanism in which the 5′-deoxyadenosyl radical abstracts a H-atom from C(2) of glycolaldehyde (or of its hydrate) could lead more directly to the *cis*-ethanesemidione radical.

Role of the Monovalent Cation. The presence of propane-1,2-diol in the solvation sphere of K^+ in the structure of dioldehydrase, together with the observation that enzymatic activity requires the presence of K^+ (or a cation of similar size), leads to the question of whether the cation plays a chemical role in the reaction mechanism. Such a role has recently been considered, assuming that the cation remains associated with the radical intermediates in all steps of the transformation [12]. The monovalent cation is also required for suicide inactivation of dioldehydrase by glycolaldehyde or 2-chloroacetaldehyde. If the required cation participates in the reaction with substrates, it may also participate in suicide inactivation. The present results show that the Tl^+ ion, which activates the catalytic reaction leading to suicide inactivation, does not interact magnetically with the *cis*-ethanesemidione radical in the inactivation complex and is unlikely to be close (within a few Å) to the unpaired electron. It may be concluded that either the cation does not directly participate in suicide inactivation or the inactivation process must lead to the dissociation of the cation from *cis*-ethanesemidione.

Final conclusions regarding the participation of the monovalent cation in the chemical mechanism of the reactions of substrates cannot be drawn from the present results. It is possible that the loss of cation interaction is part of the inactivation process in the reactions of suicide inactivators. Further experiments concerning the interactions of cations with substrates and intermediates are indicated for the future.

Experimental Part

General. [$^2H_3$]Glycolaldehyde was synthesized as described previously [7a]. Glycolaldehyde, 2-chloroacetaldehyde, D_2O, AcOTl, AcONa, and 1-methylpiperidine were from *Aldrich*. Adenosylcobalamin, AcOK, EPPS (= 3-[4-(2-hydroxyethyl)piperazin-1-yl]propanesulfonic acid), and Brij 35 (= poly(oxyethylene) lauryl ether) were from *Sigma*. *Slide-A-Lyzer* dialysis cassettes were from *Pierce*. The genes encoding the subunits of dioldehydrase from *Salmonella typhimurium* were expressed in *Escherichia coli*, and the apoenzyme was purified (specific activity: 48 units mg^{-1} at 27°) as described previously [13]. Samples containing adenosylcobalamin were maintained in dim light to avoid photolysis. Unless otherwise described, no steps were taken to exclude O_2 from the samples. EPR Specra were recorded at X-band (*ca.* 9.1 GHz) on a *Varian E-3* spectrometer; samples were maintained at 77 K, using a standard liquid-nitrogen-immersion *Dewar*. Absorbance spectra were obtained on a *Cary 50* UV/VIS spectrophotometer. ESEEM and ENDOR Spectra were collected in the pulsed EPR facility at the Arizona State University, employing a *Bruker E580* pulsed FT spectrometer, operating at 9.6 GHz and at a sample temp. of 20 K.

Preparation of Potassium-Free Dioldehydrase. Dioldehydrase was thawed on ice and augmented with Brij 35 to 0.5%. Samples were inserted into dialysis cassettes and dialyzed against buffer A (50 mM EPPS at pH 8.0, prepared with freshly distilled 1-methylpiperidine and 0.5% Brij 35) or buffer B (50 mM EPPS at pH 8.0, prepared with freshly distilled 1-methylpiperidine, 0.5% Brij 35, and 125 mM 1,2-propanediol) for 24 h. Dialysis of 0.5–2-ml samples were proceeded against 100 ml of buffer with seven exchanges. Upon completion, the samples were removed from the dialysis cassettes and augmented with Brij 35 to 0.5%. Where noted, the buffer was degassed and flushed with N_2 several times, stored under Ar, and dialysis proceeded under positive pressure of N_2. Samples were tested by ultra-low K^+ analysis at the *Wisconsin State Laboratory of Hygiene* by flame atomic-absorption spectrophotometry. In each case, trace K^+ contamination was less then 2% based on the number of active sites.

Preparation of 2-Chloracetaldehyde- and Glycolaldehyde-Inactivated Dioldehydrase for EPR Analysis. Dioldehydrase (0.20 ml, 140 μM active sites), in stock buffer (50 mM potassium phosphate, pH 8.0, 125 mM propane-1,2-diol, and 12 mM sodium cholate) was augmented with 25 μl of KCl (2M) and adenosylcobalamin (6.5 μl, 12 mM). Samples were incubated for 5 min at r.t. in the dark. After incubation, 12.5 μl of 100 mM glycolaldehyde or freshly distilled 2-chloroacetaldehyde was added to the samples. After a second 5-min incubation in the dark, samples were inserted into an EPR tube and frozen in cold isopentane/liquid nitrogen.

Preparation of Glycolaldehyde-Inactivated Dioldehydrase Containing Specific Monovalent Metal Cations in D_2O. Samples of dioldehydrase were dialyzed against buffer B and prepared, free of activating monovalent cations, as described above. Dioldehydrase (250 μl, 270 μM active sites) was augmented with AcOK, AcOTI, or AcONa, added to the concentrations stated in *Fig. 3*. Samples were then lyophilized for 1.5 h, and resuspended in 0.20 ml of D_2O. Samples were incubated with 12 μl of 5 mM adenosylcobalamin (prepared in D_2O). Samples were kept at r.t. and in the dark for 5 min. [$^2H_3$]Glycolaldehyde was added to the samples (10 μl, 80 mM) and incubated for another 5 min at r.t. in the dark. Samples were inserted into an EPR tube and frozen in cold isopentane/liquid nitrogen.

Preparation of Glycolaldehyde-Inactivated Dioldehydrase for ENDOR and ESEEM Experiments. Samples of dioldehydrase were dialyzed against buffer B and prepared, free of activating monovalent cations, as described above. Dioldehydrase (0.20 ml, 270 μM active sites) was made with 0.5% Brij 35, and augmented with 10 μl of 120 mM AcOTl or AcOK. The sample was mixed with 10 μl of 5 mM adenosylcobalamin and incubated for 5 min at r.t. in the dark. The samples were then augmented with 10 μl of 80 mM [$^2H_3$]glycolaldehyde and incubated under the same conditions for another 5 min. The samples were then inserted into EPR tubes and frozen in cold isopentane/liquid nitrogen.

Determination of Potassium Dependence of Suicide Inactivation of Dioldehydrase. Samples were dialyzed against buffer A and prepared, free of activating monovalent cations, as described above. Efforts to exclude O_2 were taken as described above. Dioldehydrase (80 μM active sites) in buffer A was added to a cuvette and

combined with 50 µl of 1 mM adenosylcobalamin. The cuvette was placed in the *Cary 50* spectrophotometer and scanned repeatedly from 350–550 nm. The absorbance was monitored for 2 min, and a diagnostic spectral trace for adenosylcobalamin was observed. Glycolaldehyde was added to the sample to a final concentration of 7.5 mM and mixed. No change in the absorbtion spectra was observed for 2 min. Then, KCl was added to the cuvette to a final concentration of 25 mM and mixed. The absorbance spectrum quickly changed to a trace, diagnostic of the formation of cob(II)alamin. The same experiment, repeated with 2-chloroacetaldehyde in place of glycolaldehyde, gave similar results.

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