Revisitation of the $\beta$CI-Elimination Reaction of d-Amino Acid Oxidase

**NEW INTERPRETATION OF THE REACTION THAT SPARKED FLAVOPROTEIN DEHYDROGENATION MECHANISMS**

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D-Amino acid oxidase (DAAO) from pig has been reported to catalyze the $\beta$-elimination of CI$^-$ from $\beta$CI-d-alanine via abstraction of the substrate $\alpha$H as H$^+$ (“carbanion mechanism”) (Walsh, C. T., Schonbrunn, A., and Abeles, R. H. (1971) *J. Biol. Chem.* 246, 6855–6866). In view of the fundamental mechanistic importance of this reaction and of the recent reinterpretation of the DAAO dehydrogenation step as occurring via a hydride mechanism, we reinterpreted the elimination reaction using yeast DAAO. That enzyme catalyzes the same reactions as the pig enzyme but with a much higher efficiency and a substantially different kinetic behavior. The reaction is initiated by a very rapid and fully reversible dehydrogenation step. This leads to an equilibrium ($k_{\text{on}} = k_{\text{reverse}}$) between the complexes of oxidized enzyme-$\beta$CI-d-alanine and reduced enzyme-$\beta$CI-iminopyruvate. In the presence of O$_2$ the latter complex can partition between an oxidative half-reaction and elimination of CI$^-$, which proceeds at a rate of $\approx$50 s$^{-1}$. This step forms a complex between oxidized enzyme and enamine that is characterized by a charge transfer absorption (which describes its rates of formation and decay). A minimal scheme that lists relevant steps of the reductive and oxidative half-reactions and elimination pathways along with the estimate of the corresponding rate constants is presented. $\beta$-Elimination of CI$^-$ is proposed to originate at the locus of the enzyme-$\beta$CI-iminopyruvate complex. A chemical mechanism that can account for elimination is discussed in detail.

In their seminal paper that appeared in this journal in 1971, Walsh *et al.* (1) reported that upon incubation with d-amino acid oxidase (DAAO)$^{2}$ (EC 1.4.3.3) $\beta$CI-d-alanine ($\beta$CI-d-Ala) eliminates CI$^-$, which was later confirmed by others (2–4). Based on this, the mechanism depicted in Scheme 1 for the elimination reaction was put forward (1). In this reaction the substrate $\alpha$H as H$^+$ is abstracted to form a carbanion intermediate, which then releases CI$^-$ to yield the final products pyruvate and NH$_4^+$. In this reaction the redox state of the flavin cofactor remains unaltered.

“Normal” dehydrogenation of $\beta$CI-d-Ala would yield $\beta$CI-pyruvate ($\beta$CI-Py) as the final product and lead to reduction of the flavin cofactor (5). The initial experiment (1) provided the first clues for formulating a general concept for the mechanism underlying flavin dehydrogenation and represents the birth of the so-called carbanion mechanism. This concept then gained wide acceptance for decades to come. However, some doubts soon emerged (2, 6–8); experiments by Hersh and Jorns (9) demonstrated that for a DAAO in which the flavin cofactor was replaced by 5-deaza-flavin, CI$^-$ was not eliminated. This puzzle remained unanswered for a long time, as the flavin ought not to be involved directly in elimination. Later experiments based on the concept of the linear free energy relationship were not in favor of the carbanion mechanism either (10, 11). A way out of the conundrum emerged when the three-dimensional structure of two related DAAOs was identified (12, 13); these studies showed unambiguously that there is no functional group at the active center of the enzyme that could act as a base in abstracting the substrate $\alpha$H as H$^+$. Furthermore, the structures of complexes of *Rhodotorula gracilis* d-amino acid oxidase (RgDAAO) with d-alanine or d-CF$_3$-alanine show that the substrate $\alpha$C-H points directly toward (the Lowest Unoccupied Molecular Orbital, of) the flavin N(5) and is thus poised for hydride transfer (13). The various arguments in favor of a hydride transfer mechanism for flavin-mediated dehydrogenation reactions have been discussed extensively elsewhere (14, 15). In the meantime, experimental evidence from a variety of flavoproteins has converged at the “hydride transfer” mechanism and shows it to be generally valid (14). Despite this, the carbanion mechanism is still presented to a general audience as the mechanism of choice, e.g. in modern biochemistry textbooks.

Although the mechanistic issue (carbanion versus hydride transfer mechanisms) might be regarded as being solved, the basic question as to how CI$^-$ is eliminated is still open. This is of cardinal importance for two reasons; (i) because the hydride mechanism as such cannot induce elimination, it assumes that elimination proceeds via a different mechanism for which the flavoprotein must possess intrinsic prerequisites, and (ii)
strictly speaking, a mechanism cannot be generally valid if it fails to provide a rationale for a connected experimental observation.

Therefore, we set out to provide an answer to the aforementioned unresolved questions. We investigated the β-elimination reaction using some modern methods and, in particular, using RgDAAO. The kinetic behavior of the latter DAAO differs from that of the enzyme from pig (pkDAAO) (16); the substrate dehydrogenation step is substantially faster, and the rate-limiting step is different. This has turned out to be the key for the mechanistic interpretations presented here.

EXPERIMENTAL PROCEDURES

Enzymes and Buffers—D-Amino acid oxidase from R. gracilis was produced and purified from recombinant BL21(DE3)pLysS E. coli cells carrying the pT7-DAAO expression plasmid as reported by Molla et al. (17). Composite buffer was 15 mM boric acid, 15 mM phosphoric acid, 15 mM sodium carbonate anhydrous, 1% glycerol, adjusted to the desired pH with NaOH, with a working pH range of 6.0–9.0.

Absorption Measurements and Pyruvate Molar Extinction Coefficient—Enzyme concentration is indicated in terms of flavin content using an ε_{455 nm} = 12,600 M^{-1} cm^{-1} (16, 17). The pyruvate molar extinction coefficient at 320 nm was determined as 20 ± 1 M^{-1} cm^{-1} by linear fitting of the absorbance values of samples containing increasing, known concentrations of pyruvate in composite buffer, pH 7.0, at 25 °C.

NMR Spectra—A JEOL (GX 400) 400-MHz instrument was used. Samples were ≈0.5 ml in 5-mm tubes, and measurements were performed at 28 °C; 8–16 pulses were recorded and averaged for Fourier transformation. Spectra evaluation and transformation was performed with MestReC.

Rapid Reaction (Stopped Flow) Measurements—Rapid reaction measurements were carried out in composite buffer at 25 °C using a stopped-flow spectrophotometer equipped with a thermostat and a diode array detector (J&M Analytische Mess- und Regeltechnik GmbH) as detailed (18–20). All concentrations mentioned in these experiments refer to those after mixing. See the supplemental material “Additions to Experimental Procedures” section for further conditions. The rate of chloride release was assessed by measuring the change in pH as detailed in the legend to Fig. 10. Lactate and lactate oxidase were added as an O2 scavenger system.

Kinetic Isotope Effects—Buffer and substrate solutions for solvent kinetic isotope effects (KIE) studies were prepared by dissolving the appropriate reagents in D2O. Concentrated enzyme stock solutions in H2O were diluted into D2O buffers such that the final proportion of D2O was 95%. For details, see Harris et al. (19). Primary KIEs were measured with 3H-βCl-D,L-Ala either in 100% H2O or 95% D2O.

HPLC Analysis—HPLC was performed as described earlier (21).

RESULTS

The β-Elimination from βCl-D-Ala Produces Pyruvate and Cl-Pyruvate in the Presence of O2

We studied the reaction with RgDAAO in the pH range from 6 to 10, whereas Walsh et al. (1) conducted the reaction at pH 8.5 using pkDAAO. With respect to product formation, qualitatively similar pictures emerge for both DAAOs, but important and substantial differences are also apparent. In view of the shortcomings of the method used by Walsh et al. (Ref. 1; derivatization using 2,4-dinitrophenylhydrazine and thiosemicarbazide), we followed the disappearance of βCl-D-Ala and the formation of pyruvate and that of other products by HPLC, as described in Gibson et al. (22). In this way the amounts of involved species can be estimated as a function of time. A representative time course of formation of pyruvate and disappearance of βCl-D-Ala is depicted in Fig. 1 for the reaction conducted at pH 7.0.

At pH 7.0 and in the presence of ≈1 mM O2 (100% saturation), formation of pyruvate (Py) and the disappearance of βCl-D-Ala proceed at identical rates, suggesting that the two processes are directly kinetically linked (Fig. 1). Under these specific conditions (βCl-D-Ala = 20 mM) an estimated 85% of the βCl-D-Ala is converted to pyruvate via Cl− elimination. This contrasts sharply with the case of pkDAAO in which close to 100% βCl-Py is formed at 100% O2 saturation (i.e. no elimination occurs) (1). It should be noted here that the formation of HCl continuously lowers the pH of the reaction mixture, which affects the course of the reaction and, at pH < 7, induces enzyme denaturation. The relative amount of Py formed (corresponding to the elimination reaction) is fairly constant from pH 6 to 7; however, it decreases substantially at pH ≥ 8 (not shown, see also Fig. 4). A second, primary product formed in the presence of O2 is βCl-Py (1). βCl-Py in aqueous solution is
assumed to exist mainly in its hydrated form and so far has not been possible to attribute to a specific peak or to estimate the quantity of primarily formed Cl-Pyruvate and pyruvate cannot be determined by correlating it to the signal denoted by ? is discussed in the supplemental material, “Comments to NMR Experiments” section.

**Estimation of the Ratio of Cl-Pyruvate Formation versus Cl− at pH 8**

From the aforementioned description (see also supplemental material, “Comments to NMR Experiments” section) it is evident that amounts of βCl-Py and pyruvate cannot be determined by using direct methods. We thus attempted to estimate the quantity of primarily formed βCl-Py by correlating it to the consumption of βCl-d-Ala and dioxygen. This is based on the stoichiometry of the two competing reactions as shown in Equations 1a and 1b) (see below and the legend to Fig. 4 for details).

\[
E_{\text{ox}} + \beta \text{Cl-d-Ala} + O_2 \rightarrow (E_{\text{red}} + \beta \text{Cl-Py} + NH_3 + O_2)
\]

\[
E_{\text{ox}} + \beta \text{Cl-Py} + H_2O_2 + NH_3 \quad \text{(Eq. 1a)}
\]

\[
E_{\text{ox}} + \beta \text{Cl-d-Ala} \rightarrow E_{\text{ox}} + Cl^- + Py \quad \text{(Eq. 1b)}
\]

Equation 1a is the normal dehydrogenation reaction in which 1 eq O₂ is consumed/molecule of βCl-d-Ala to form 1 eq of βCl-Py and H₂O₂, whereas Equation 1b is the β-elimination reaction that consumes βCl-d-Ala but no O₂.

For this experiment we adapted the method of Gibson et al. (22) in which the time dependence of the oxidation state of a flavoprotein (which changes with time according to the reaction in Equation 1a) is monitored during turnover by using its absorbance at 450 nm; see also Refs 17, 20, and 23. In the specific experiment (Fig. 3) Abs₄₅₀ nm decreases very rapidly immediately upon mixing the reactants, corresponding to an apparent, partial reduction of the enzyme. As a result and for up to 40–60 s, the system enters a stationary (turnover) phase in which Eₙox and Eₙred (oxidized and reduced enzyme forms) are present in a ratio of 3–5:1. This is deduced based on the value of “start” ~100% Eₙox and the end absorbance obtained with βCl-d-Ala = 2 mM, which corresponds to that of Eₙred. After the stationary phase, the residual O₂ concentration > βCl-d-Ala concentration, Eₙox is (re)formed. This is the case when the starting βCl-d-Ala is ≤1.0 mM. When βCl-d-Ala is present in sufficiently large excess over O₂, the latter becomes exhausted, and RgDAAO is eventually fully converted to the Eₙred form. This occurs when the starting concentration βCl-d-Ala = 2 mM (Fig. 3). From this it can be estimated (see inset of Fig. 3) that at βCl-d-Ala ~1.5 mM the system would end up in an intermediate situation where Eₙox and Eₙred remain unaltered over time. Under these conditions, ~0.25 mM (= initial O₂) out of 1.5 mM

**βCl Elimination by Yeast DAAO**

**FIGURE 2. NMR spectrum of the incubation of βCl-d-Ala with RgDAAO at pD 7.3.** The NMR spectra were recorded as detailed under “Experimental Procedures.” Conditions were ~500 µl of 0.1 M potassium phosphate buffer in D₂O (pD 7.3) containing 2 µM RgDAAO, 20 mM βCl-d-Ala, and 20 µM aceta-mide (CH₃CONH₂) as internal standard (30 °C). The NMR tube was flushed gently with 100% O₂ before adding RgDAAO and shaken 2–3 times between the individual measurements. The spectra shown were recorded after ~50–60 min of incubation (average of 8 pulses). The insets show expanded spectral segments for acetate (right panel), pyruvate (central panel), and the area between 3.5 and 4.3 ppm. In this section some residual Cl-Py was a main primary product; while lower amounts, as estimated from proton integration, were detected than would be expected if Cl-Py but likely products resulting from secondary reactions. This is based on the stoichiometry of the two competing reactions as shown in Equations 1a and 1b) (see below and the legend to Fig. 4 for details).

**FIGURE 3. Estimation of the ratio of Cl− β-elimination versus formation of βCl-Py.** The enzyme, 5 µM in composite buffer pH 8.0, was reacted at 25 °C in the stopped-flow instrument with the mM concentrations of βCl-d-Ala indicated in square brackets (final concentrations) and at O₂ = ~0.25 mM. Note that start corresponds to the absorbance of 100% oxidized enzyme (Eₙox) and that at ~80 s and with 2.0 mM βCl-d-Ala, fully reduced enzyme (Eₙred) is formed. In the main panel, trace (- -) represents an estimated absorbance value (~0.06) on the ordinate that corresponds to the starting βCl-d-Ala where Eₙox and Eₙred would remain constant with time at ~40 s upon exhaustion of both βCl-d-Ala and O₂. The inset shows the 450-nm absorbance observed at 40 s and at the indicated βCl-d-Ala; therein the dotted lines indicate that with an initial βCl-d-Ala ~1.5 mM both reactants (βCl-d-Ala or O₂) are exhausted at the end of the reaction. See text for further details, “Estimation of the Ratio of Cl-Pyruvate Formation versus Cl− at pH 8” section.
βCl-D-Ala would have been converted via normal, oxidative turnover, whereas ≈1.25 mM would have undergone Cl⁻ elimination. The ratio of pathways 1b/1a can thus be estimated as ≈5, and \( k_{\text{elim}} \approx 5 \times k_{\text{red}} \) in Eq. 1a, under the specific conditions of Fig. 3 (k is the apparent rate constant for the dehydrogenation reaction as reported in Eq. 1a), where βCl-D-Ala concentration is in the same range as O₂ concentration. The latter cautionary statement is necessary because the reaction with dioxygen contains a second-order term whose rate constant can only be estimated as \( \approx 10^{-6} \text{ M}^{-1} \text{s}^{-1} \) (see below) (16). This experiment demonstrates the competitive behavior of oxidative and elimination pathways and their dependence on the ratio of the reagent concentrations.

Incorporation of the α⁺H of βCl-o-Ala into Pyruvate

An intriguing feature of the elimination reaction is the (partial) retention of labeled α⁺H of βCl-α⁺H-D-Ala in β-position of the ketoacid product, an issue that has to be taken into account for formulating alternate mechanisms (see Equation 2) (1, 4). With βCl-α⁺H-D-Ala and pkDAAO at pH 8.5, a 20–40% label retention was found (1, 4). With RgDAAO we carried out the elimination reaction in a NMR tube using βCl-α⁺H-D,L-Ala or βCl-α⁺H-D-Ala in D₂O (Fig. 4). It should be pointed out that, as Walsh et al. (1) also noticed, the rate of the reaction progressively slows down with time, whereas repeated equilibration with O₂ (shaking with air) restores elimination activity.

The conversion was followed until ≈90% of the βCl-D-Ala was consumed. In the NMR spectra CH₃- and CH₂ pyruvate are easily discerned (Fig. 4). CH₃-pyruvate shows a narrow singlet at 2.360 ppm, whereas CH₂D-pyruvate exhibits a (1/1/1) triplet centered at ≈2.344 ppm. The fine structure of this latter signal results from the 1H₂-2H coupling in the CH₂ ²H group. Integration of the signals from the reaction of βCl-β[¹H]Ala in D₂O at pD 7.3 indicates that out of the total hydrogens (¹H), ≈50% are in CH₃⁻, and ≈50% are in CH₂D-pyruvate, i.e. 3 H versus 2 H, respectively, thus yielding a ratio of 50/3 versus 50/2 for CH₃/CH₂D = 2/3 in the product pyruvate. When the reaction is started from βCl-D,L-α⁺H-Ala, essentially only CH₃D-pyruvate is produced. From this it follows that at pD 7.3 the extent of α⁺H label “loss” is ≈60%, with the remaining ≈40% corresponding to retention. Here we want to stress that possible KIEs were not being considered in these estimates. The reaction at pD 8.3 and 9.3 is more complex as the “transient inactivation phenomenon” (1) is more pronounced and requires repeated equilibration with air to attain ≈90% conversion. At pD 8.3 and 9.3 a similar amount of retention is found (see Fig. 4). An analogous experiment was carried out using βCl-D,L-²H-Ala in H₂O at pH 8.0. The integration of the signals corresponding to CH₃⁻ and CH₂D-pyruvate was ≈55 and 45% (supplemental Fig. S2B), suggesting that solvent-borne ²H was incorporated in the product to a somewhat larger extent than for incubations of βCl-D-[¹H]Ala in D₂O (compare black full line traces in Fig. 4 and supplemental Fig. S2B). The ≈40% of label incorporation at pD 7.3 with RgDAAO compared to the 20–40% reported by Walsh et al. (1) for pkDAAO. We thus repeated the incubation procedure in a NMR tube under conditions similar to those described in Walsh et al. (1), i.e. using pkDAAO at pD 8.6 and with 100% O₂; the extent of retention is ≈20%. It thus appears that the extent of loss of the H label with solvent is only marginally dependent (i.e. within the margin of error) on conditions such as pH, solvent, source of enzyme, and O₂ concentration and on whether the reaction is started from βCl-D,L-²H-Ala or βCl-D-[¹H]Ala. In other words the extent of label retention is likely to be associated with a mechanistic feature of the elimination reaction itself. In the NMR experiments only traces of signals could be observed that might be attributed to βCl-Py in its hydrated form (see supplemental Fig. S2 and comments therein). On the other hand and in particular at pD 8.3 and 9.3, several complex signals appear in the region 3.2–4.7 ppm concomitantly with the disappearance of the peaks belonging to βCl-D-Ala. Their chemical shifts and patterns sug-
suggest they belong to unidentified products of secondary reactions of BCI-Py, probably with residual BCI-d-Ala.

Kinetic Studies

Two Charge Transfer (CT) Intermediates Are Detected during the Course of the Reaction—A detailed study of the spectral course of the reaction of RgDAAO with BCI-d-Ala was carried out at pH 6–9 using the stopped-flow instrument (see “Experimental Procedures”). At pH 8 (supplemental Fig. S3A) and 9 (Fig. 5) the courses are very similar, although at pH 9 the various intermediates are distinguished best. This is shown in Fig. 5 where the first spectrum was recorded at 0.8 ms upon mixing the reactants. This spectrum is characterized by an ∼50% decrease in the original absorption of the oxidized flavin in the 450-nm region. Importantly, the phase leading to this spectrum occurs almost completely during the “dead time” of the instrument and is just about completed at 2–3 ms (see the traces in Fig. 5 and supplemental Fig. S3). Concomitantly, a long wavelength absorbance forms at >520 nm that is attributed to a CT complex. This species (named intermediate 1 charge transfer (I1-CT)) presumably consists of a mixture of chromophores derived from oxidized and reduced flavin in a ratio ∼1:1. This deduction is based on the observed ratio of the 440-nm absorbance of oxidized (spectrum recorded at 0 ms) and reduced enzyme (spectrum recorded at 12 s). I1-CT then converts to a second charge transfer species (I2-CT) with higher absorbance both in the 440- and 550–650-nm region. This species attains maximal absorbance at ∼100 ms (Fig. 5), where the system enters a short stationary phase. At pH 8, 7, and 6, a qualitatively similar behavior is observed (see the panels of supplemental Fig. S3), although the intensities of the corresponding species are significantly different. The absorption spectrum of I2-CT (Fig. 5) suggests that its main component is oxidized enzyme flavin in complex with an electron donor, which gives rise to the CT absorption observed at >520 nm. The time dependence of the absorbances was also analyzed using the application SpecFit with which the spectra of intermediates can be identified in sequential processes (see the supplemental material “Additions to Experimental Procedures” section for details); the spectra obtained by this deconvolution procedure for I1-CT and I2-CT can be superimposed on those recorded directly (see Fig. 5). A decrease in the absorbance of I2-CT then ensues. It leads at ∼12 s to a final species with a spectrum that is closely similar to that of free reduced RgDAAO (16), this occurring concomitantly with oxygen consumption in the system. Notably, the extent of absorbance increase in the 320-nm region at pH 9 (∼0.05 absorbance units) is small compared with that of the same experiment at pH 6 (supplemental Fig. S3C). This is consistent with formation of small amounts of pyruvate at pH 9 (∼2.5 mM from 100 mM BCI-d-Ala in the experiment of Fig. 5) in contrast to substantial amounts at pH 6 (supplemental Fig. S3C).

The β-Elimination Reaction at pH 6 in the Presence of O2—Although basically the same products are formed as at pH 7, 8, or 9, the spectral course of the reaction at pH 6 (supplemental Fig. S3C) under similar conditions of reactant concentrations is substantially different from the former one (see Fig. 5 for pH 9) as demonstrated by the following observations; (i) the decrease in absorbance in the 450-nm region that occurs during the dead time of the instrument and corresponds to the formation of I1-CT is ∼10% compared with ∼50% at pH 9 (Fig. 5) or 8 (supplemental Fig. S3A), (ii) I1-CT is formed at a slower rate, its maximal formation is at ∼2–3 ms (supplemental Fig. S3C), and it is also converted at a much slower rate into I2-CT, (iii) the reaction takes >10 times longer to reach completion, (iv) at that point the enzyme exists largely in the oxidized state, (v) the amount of pyruvate formed is much larger, as reflected by the absorbance increase at 320 nm (inset of supplemental Fig. S3C), and (vi) the steady-state phase encompasses ≥100 s compared with 3–4 s at pH 9.

The β-Elimination Reaction at pH 8 in the Absence of O2—The course of the anaerobic reaction is similar at pH 9 and 8 (supplemental Fig. S4), the latter conditions corresponding to those used by Walsh et al. (1) under which pyruvate was reported to be formed exclusively. In the present case only <20% of the possible amount of pyruvate is formed (see the time course of absorbance at 320 nm in the inset of supplemental Fig. S4 and compare with the inset to Fig. 5). Drastic differences can also be observed when comparing the spectral courses of the pkDAAO (1) and RgDAAO incubations. Fig. 2C in Walsh et al. (1) shows that during the “steady-state” phase, a species is present that contains predominantly oxidized pkDAAO and exhibits a CT absorption. At the end of the incubation, essentially all pkDAAO was in the oxidized state (1). In the present case with RgDAAO, the spectral course of the corresponding reaction is depicted in supplemental Fig. S4 and shows that the enzyme is in the reduced state at the end of the incubation.
The initial events observed under anaerobic conditions are similar to what is observed in the presence of oxygen. Thus, the very first spectrum (supplemental Fig. S4) obtained at 0.8 ms upon mixing the reactants in the stopped-flow instrument reflects an ~25% decrease in the 440-nm band of the oxidized enzyme that occurs during the dead time of the instrument. This initial decrease is smaller than that found in the presence of O$_2$ (~40%, Fig. 5), which is a counterintuitive observation. A concomitant increase occurs in the 530–600-nm region that reflects the formation of a charge transfer complex (I1-CT) similar to that observed under aerobic conditions (Fig. 5). From ~70 ms and up to 300–400 ms the system enters a steady-state situation (see supplemental Fig. S4, inset) that is much shorter than that observed under aerobic conditions (Fig. 5) and that gradually leads to the final species within ~30 s. There are two relevant differences between the behavior of RgDAAO and that of pkDAAO (1); with RgDAAO the spectral features of the final species are consistent with formation of fully reduced enzyme flavin. By comparison, at the end of the reactions and after approximately the same incubation time under similar conditions, pkDAAO ends up in the fully oxidized state (4). A second difference pertains to the quantity of pyruvate formed. This is ~0.1 eq of the available βCl-D-Ala with pkDAAO (estimated by the increase in absorbance at 320 nm, see supplemental Fig. S4, inset), whereas with pkDAAO up to 0.5 eq are formed (1).

As reported for pkDAAO (1), a progressive loss of activity with incubation time was also observed with RgDAAO; this is apparent from the 320-nm trace in the inset of supplemental Fig. S4. Elimination activity can be restored by admitting oxygen. The exact reason for this behavior is still elusive, but we suggest that during the anaerobic incubation a less reactive, reduced flavin form accumulates that is (re)converted to oxidized enzyme (active in β-elimination) upon reaction with dioxygen.

**Kinetic Course of the Cl$^-$ Elimination Reaction**

Evidence for Full Reversibility of the Initial Redox Step—In rapid mixing experiments such as those shown in Fig. 5, I1-CT, the very first observable species is formed almost completely during the dead time of the instrument. Fig. 6 shows that the decrease in absorbance recorded at 0.8 ms depends on the concentration of βCl-D-Ala. In Fig. 6, the trace 5 is representative and was obtained at 50 mM βCl-D-Ala. The inset to Fig. 6 correlates the extent of the absorbance decrease at 450 nm occurring in the dead time and the extent of formation of I1-CT with the concentration of βCl-D-Ala. This is a typical situation in which the reactants very rapidly form a complex that reacts reversibly to reach an equilibrium (24), as represented by Equation 3, where $K_d$ is the dissociation constant for the rapid equilibrium of βCl-D-Ala binding, $k_f$ and $k_r$ are “forward” and “reverse” steps for the ensuing redox reaction, and $E_{red}^{-}Cl$-IPy is the complex of reduced enzyme with the product βCl-IPy. In a situation such as that in Fig. 6, an apparent dissociation constant $K_{d,app} = K_d \times (k_f/k_r) \approx 5$ mM can be estimated from the plots in the inset of Fig. 6. The concentration ratio of the species $E_{ox}^{-}Cl$-D-Ala/$E_{red}^{-}Cl$-IPy and that of the steps $k_f/k_r$ that link these species can be deduced from the spectra shown in Fig. 6 as follows; the spectrum of $E_{ox}^{-}Cl$-D-Ala is taken as being not relevantly different from that of uncomplexed $E_{ox}^{-}$ (16), and the spectrum of $E_{red}^{-}Cl$-IPy (trace A in Fig. 6) is obtained by deconvolution with the application SpecFit. From this it is estimated that the ratios $E_{ox}^{-}Cl$-D-Ala/$E_{red}^{-}Cl$-IPy and $k_f/k_r$ are approximately 2/3 under substrate saturation conditions. Consequently the reaction up to I1-CT constitutes a (fast) approach to the equilibrium depicted in Equation 3. Because $k_f/k_r \approx 2/3$, the dissociation constant $K_d$ for the formation of the encounter complex $E_{ox}^{-}Cl$-D-Ala can be estimated as ~3 mM. At pH 9.0 the situation is similar, the equilibrium being also slightly in favor of the reduced species (not shown). At pH 7 the same equilibrium is ~1:1, whereas at pH 6 and under saturating conditions $E_{ox}^{-}Cl$-D-Ala/$E_{red}^{-}Cl$-IPy ~4:1 (not shown, compare with supplemental Fig. S3C).

Comparison of Reaction Courses under Aerobic and Anaerobic Conditions—The shapes of the spectra of I1-CT are not significantly affected by the presence of O$_2$, although their intensities and specifically the ratio of the 450/550 nm absorbance differ (supplemental Figs. S3A and S4). I2-CT is formed at similar rates from I1-CT and also attains a maximum at ~100 ms both in the presence and absence of O$_2$ (supplemental Fig. S5). At longer time scales the absorption versus time profiles are
very different because in the absence of O₂, the enzyme is rapidly converted to the reduced form (supplemental Fig. S5), whereas under aerobic conditions the system enters a steady-state phase and takes longer to convert to the reduced form, which occurs upon exhaustion of O₂ at 15–20 s.

Estimation of Rate Constants of I₁-CT Conversions into I₂-CT—Based on the aforementioned approach (supplemental Fig. S5), rates of I₁-CT conversions into I₂-CT were estimated. The results are depicted in Fig. 7 as a function of the rates of I₁-CT conversions into I₂-CT were estimated. The k₅ and k₆ was found to be consistent with the substrate binding step being concentration for pH 8. At all pH values saturation behavior was found to be consistent with the substrate binding step being

Estimation of the Rate of the Product Enamine Dissociation from Oxidized RgDAAO—Because elimination should proceed via formation of an enamine from βCl-D-Ala, it was reasoned that this enamine is the species that gives rise to a CT band in a complex with oxidized flavin enzyme. In this complex the enamine should exist in its anionic (unprotonated amine) state and serve as the donor as a protonated αN would most likely not have such a capacity. As also discussed below, intermediate I₂-CT is thus proposed to be this Eox-enamine complex (see Scheme 2). The rate of dissociation of this complex to yield free Eox was estimated in a double stopped-flow experiment as shown in Fig. 8. The rationale behind the experiment is that the good ligand benzoate (a competitive inhibitor) (23) effectively traps Eox upon its formation (see Scheme 2) and thus impedes any further cycling of the enzymatic elimination reaction. The disappearance of the CT band with a rate = 26 s⁻¹ thus likely corresponds to enamine release, I₂-CT thus being the Eox-enamine complex (see Scheme 2).

FIGURE 7. Dependence of rate of conversion of I₁-CT into I₂-CT from the βCl-D-Ala concentration at pH 8.0. The rate constants were obtained from global analysis of primary data such as those in Fig. 5 and as detailed under “Experimental Procedures.” The data points are the average of 4–6 individual measurements, and the vertical bars indicate their scatter. The lines through the data points are fits based on the pre-equilibrium equation, including a reverse step k₅ that yields an intercept on the ordinate (aerobic: k₅ 77 ± 4 s⁻¹; k₆ = 8 ± 3 s⁻¹, R² = 0.977; anaerobic: k₅ 68 ± 1 s⁻¹, k₆ = 4 ± 0.2 s⁻¹, R² = 0.999; apparent k₅ 12 and 16 mM, respectively).

SCHEME 2. Kinetic minimal scheme for the reaction of RgDAAO with βCl-D-Ala. The scheme is subdivided in four parts. The part above the gray bar refers to enzyme species in which the flavin is in its oxidized state, the lower part refers to enzyme species containing reduced flavin. The right side encompasses processes that occur in normal catalysis, i.e., a reductive (central, vertical equilibria) and an oxidative half-reaction involving O₂. The left side shows the cycle proposed to be involved in Cl⁻ elimination. The data in red are estimates for the rates of corresponding steps. The values (*) for k₅ (≈ 1.2 × 10⁶ M⁻¹ s⁻¹), k₆, and k₇ (≈5 × 10⁴ M⁻¹ s⁻¹) are those estimated for D-Ala as substrate (16), a direct measurement not being feasible due to the reactivity of Cl-pyruvate. Steps k₅, k₆, and k₇ involve dissociation of a product as well as hydrolysis by H₂O, the two processes not being differentiated.
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Elimination Starting from Reduced Enzyme Is Associated with Flavin Reoxidation—To define the redox state at which Cl– elimination occurs, the competence of reduced RgDAAO was investigated at pH 7.0 as at this pH substantially more pyruvate forms than at higher pH values (see above). Walsh et al. (1) addressed the same question using reduced pDAAO; they reported that β-elimination did not occur. In the present case RgDAAO was first made anaerobic and subsequently converted to the reduced state with a small amount of D-Ala (∼1.5 molar excess, Fig. 9), whereby special care was taken to avoid the presence of any oxidized enzyme (see the comments in the supplemental material, “Comments on the Cl– Elimination from βCI-d-Ala Starting from Reduced Enzyme” section) Fig. 9 shows that reduced RgDAAO does indeed interact with βCI-d-Ala. The first part of the reaction, i.e. from 1 ms to 30 s, was examined with the stopped-flow instrument (supplemental Fig. S6). Up to 2 s nothing happens. However, subsequently and up to 30 s a progressive increase in absorbance in the 450- and 550-nm region ensues that corresponds to (re)oxidation and leads to formation of ∼50% of the possible quantity of oxidized enzyme (see Fig. 9) and some absorption due to a CT interaction. Concomitantly, production of pyruvate (see 320-nm trace) sets in and continues progressively. Importantly, there is a 1–2-s lag phase preceding the absorbance increases at 450 and 540 nm (supplemental Fig. S6), whereas the absorbance increase at 320 nm reflects pyruvate formation sets in at 10–20 s (Fig. 9). The maximal rate of this process corresponds to a quasi-steady-state in which the relative concentration of oxidized RgDAAO remains maximal. Presumably there is no real discrepancy between the present results and the negative ones reported by Walsh et al. (1). In the present case the concentration of DAAO is ∼10-fold higher than that used in Walsh et al. (1). Furthermore, Walsh et al. (1) used an ∼20-fold excess of reductant (substrate); consequently, any net reoxidation of their enzyme was prevented. We conclude from these experiments that reduced DAAO is not able to carry out elimination catalytically at an appreciable rate. However, a component present in the solution, possibly βCI-d-Ala, very slowly reoxidizes the reduced enzyme, thus generating the oxidized form that is competent in catalytic β-elimination.

Isotope Effects—To correlate the steps of dehydrogenation and elimination, the courses of the reactions of α3H-βCI-d-Ala and α3H-βCI-d-Ala were compared. The rates of conversion of II-CT into I2-CT at pH 8 are $k_{obs} = 97$ and 73 s$^{-1}$ for α3H- and α3H-βCI-d-Ala, corresponding to a KIEs of ∼1.3 (see the legend to supplemental Fig. S7, A and B for details of the conditions). The rate of the (re)oxidative half-reaction(s) with α3H-βCI-Ala is also slowed down as the ratio of $E_{ox}/E_{red}$ is lower, and the system requires a longer time during turnover to exhaust O2 (supplemental Fig. S7B). These effects reflect a KIE ∼1.5. Solvent KIE (supplemental Fig. S7, C and D) and corresponding proton inventory were carried out to study the involvement of solvent-borne hydrogens in the elimination process. At pH 8 and when followed by the initial rate of pyruvate production, elimination proceeds with a solvent deuterium KIE ∼1.7. Pyruvate formation/elimination is best followed at pH < 7 where it is the major process. At pH 6.5, the proton inventory of pyruvate formation shows a fairly linear profile with a KIE ∼1.7 (supplemental Fig. S7E), this being compatible with the effect...
conversion of intermediate 11-CT into 12-CT proceeds at \( \approx 35 \text{s}^{-1} \) (curves A and B, first fast phase). Curve C shows that there are no relevant absorbance changes at \( \approx 463 \text{ nm} \) (isosbestic point for the conversion of intermediate 11-CT into 12-CT) in the absence of indicator during the first reaction phase. However, in the presence of indicator an absorbance increase was observed that reflects a lowering in pH and that proceeds at \( \approx 55 \text{s}^{-1} \) (curve D). Because the rates of the preceding and of the subsequent steps differ by up to 2 orders of magnitude, it is reasonable to assume that the conversion of intermediate 11-CT into 12-CT and that of H\(^+\) release all reflect the same chemical event, namely, Cl\(^-\) elimination.

DISCUSSION

Although pkDAAO and RgDAAO share the ability to catalyze the elimination of Cl\(^-\) from \( \beta Cl\text{-d-Ala} \), substantial differences can also be seen in the respective kinetic behaviors and in particular with respect to the dependence of the process from dioxygen. Thus, in a key statement Walsh et al. (1) wrote: "... anaerobic incubations of pkDAAO with \( \beta Cl\text{-chloroalanine yield pyruvate exclusively. When similar incubations were conducted with 100\% O\(_2\) as the gas phase, the expected keto acid product, chloropyruvate, was formed almost exclusively."

The yeast RgDAAO behaves substantially different in this respect, and we have exploited this to attribute specific kinetic steps to chemical events occurring during elimination and to identify the observed intermediates.

Identification and Attribution of Kinetic Steps—Because kinetic analyses were conducted at various pH values, for the sake of clarity we focus the discussion on the most representative case of pH 8 when differences to other conditions are not relevant. Fig. 3 shows that elimination, a reaction formally not involving changes in redox states, and normal dehydrogenation (as defined by substrate dehydrogenation coupled to oxygen consumption; see also Scheme 2) are concurrent events. This is in line with previous deductions (1, 26) and is depicted in more detail in Scheme 2. In that scheme, the two processes share the species \( E_{ox} \) and \( E_{red}\beta Cl\text{-Py} \). Catalysis starts with fast and fully reversible binding of \( \beta Cl\text{-d-Ala} \) to form the \( E_{ox}\beta Cl\text{-d-Ala} \) complex via steps \( k_{1}/k_{-1} \) and with a \( K_{d} \) (\( k_{-1}/k_{1} \approx 3 \text{ms}^{-1} \)) that is similar to that for d-Ala (16, 19). This conclusion is derived from experiments such as those of Figs. 6 and 7 that show saturation behavior of the observed initial steps (\( k_{obs} \)) on \( \beta Cl\text{-d-Ala} \). Binding is followed by a very rapid reduction of \( E_{red}\beta Cl\text{-Ala} \) (Scheme 2) via step \( k_{2} \) that is essentially completed within \( \approx 1 \text{ms} \) and thus cannot be observed in the stopped-flow instrument (Figs. 5 and 6 and supplemental Fig. S3). The rate of \( k_{2} \) is thus \( 1000 \text{s}^{-1} \). Remarkably, in sharp contrast to other DAAO substrates but in analogy to phenylglycines (19), this reduction step \( k_{2} \) is fully reversible (step \( k_{-2} \)) the single steps having approximately the same value. The very first species observed spectroscopically at \( \approx 1 \text{ms} \) (11-CT, Fig. 5 and supplemental Fig. S3) thus consists of an equilibrium mixture of \( E_{ox} \) (as free \( E_{ox} \) and \( E_{ox}\beta Cl\text{-d-Ala} \)) and \( E_{red} \) (as \( E_{red}\beta Cl\text{-Py} \), Scheme 2).

The ensuing steps depend on the presence or absence of O\(_2\) (Scheme 2, compare Figs. 5 and 6 and supplemental Fig. S4). The initially formed 11-CT is converted to a second intermediate (12-CT) via \( k_{3} \) at a rate \( \approx 50–80 \text{s}^{-1} \) at pH 8 (Figs. 5 and 7).
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As shown in Fig. 7, the rate of this process is dependent on O_2 to a minor extent. The rate of the reaction of I1-CT with dioxygen, \( k_5 \), can be assumed to be similar to that of the complex of reduced enzyme with iminopyruvate \( E_{\text{red}} \)-IPy, i.e. around \( 1.2 \times 10^5 \) M \(^{-1}\) s \(^{-1}\) (16). The rates of I1-CT conversion into I2-CT depend on βCl-d-Ala (Fig. 7) and show finite intercepts on the ordinate. This deserves comment. As discussed in Strickland et al. (24), such an intercept corresponds to the reverse (or an equivalent combination) of the measured forward step, the latter representing an approach to equilibrium. However, it is chemically most unlikely that the microscopic reverse of step \( k_5 \), the elimination of Cl\(^{-}\), would play any role kinetically. We thus interpret the apparent “reversibility” as a combination of steps that lead to reformation of \( E_{\text{red}} \)-βCl-IPy. This is assumed to be achieved via steps \( k_4 \), \( k_1 \), and \( k_2 \) (Scheme 2), possibly also including steps \( k_6 \) and \( k_8 \). The values found for \( k_4 = 4-8 \) s \(^{-1}\) (Fig. 7), therefore, likely reflect a combination of the aforementioned single steps.

An estimation of the value of \( k_7 \), the dissociation of the \( E_{\text{red}} \)-βCl-IPy complex, can be obtained from the rate of disappearance of the long wavelength absorbance of the species in experiments in which O_2 is either absent or has been exhausted (see Fig. 5 and supplemental Fig. S4). The value \( \approx 2-3 \) s \(^{-1}\) is typical for the dissociation of imino acids from the corresponding complexes with reduced enzyme (16, 19). The rate of the reaction of free reduced enzyme with O_2 via \( k_8 \) is taken from the literature (16, 19). The last step in Scheme 2, the dissociation of I2-CT to form \( E_{\text{ox}} \) via \( k_8 \), was determined directly from the experiments shown in Fig. 8.

It is of crucial importance to identify the step that corresponds to the chemical event in which Cl\(^{-}\) elimination occurs. This can be deduced from the experiments of Fig. 10, according to which elimination is concomitant with step \( k_4 \), the transformation of I1-CT into I2-CT. This establishes that Cl\(^{-}\) elimination is preceded by enzyme reduction/substrate dehydrogenation, a topic that was debated in previous studies (4, 6, 27). In kinetic terms elimination reactions that would branch off at either intermediates \( E_{\text{ox}} \)-βCl-d-Ala or \( E_{\text{red}} \)-βCl-IPy (Scheme 2) would be equivalent. The overall behavior of the system can thus be described by the minimal set-up of Scheme 2 where two consecutive cycles share two intermediates, \( E_{\text{ox}} \) and \( E_{\text{red}} \)-βCl-IPy. The latter is of great importance as it constitutes the branching point for the oxidative and the elimination pathways. In this scheme the limiting step(s) for the normal, oxidative turnover cycle (Scheme 2, *right side*) is \( k_5 \) (or \( k_6 \)), whereas for the elimination pathway this is \( k_4 \), the release of the enamine product. This yields a rationale for the differences in spectral courses observed in the presence or absence of O_2 as shown in Figs. 3 and 5 and supplemental Fig. S4.

**Chemical Identity of Intermediates**—Attributing chemical entities to species I1-CT and I2-CT is of particular importance in the context of Scheme 2. The absorbance values at wavelengths >530 nm (Figs. 5 and 6 and supplemental Fig. S3) of both species are compatible with the presence of charge transfer interactions (28). I1-CT is reasonably attributed to the complex of reduced enzyme flavin with the imino acid product \( E_{\text{red}} \)-βCl-IPy, Scheme 2) in analogy to its occurrence in the reaction with normal substrates (28). I2-CT, on the other hand, exhibits the two-banded absorption of the oxidized enzyme in addition to the CT absorption at >530 nm. From this it is reasonable to assume that the oxidized flavin behaves as the acceptor in the complex. Because I2-CT is formed concomitantly with Cl\(^{-}\) elimination, the donor in the same complex would be the resulting enamine, which also ought to be in its NH\(_2\)-neutral form. This interpretation is in agreement with previous, general proposals (4, 28).

**Chemical Mechanism of Cl\(^{-}\) Elimination**—A carbanion mechanism has been excluded for the normal DAAO dehydrogenation reaction (10, 13, 29) mainly because of the absence of a functional group (base) that might abstract a H\(^+\) to form the mentioned carbanion. The validity of this argument must also apply for the Cl\(^{-}\) elimination reaction and thus speaks against a mechanism starting from an intermediate such as \( E_{\text{ox}} \)-βCl-d-Ala (Scheme 2). The kinetic data discussed above are in support of the elimination occurring “directly” from the \( E_{\text{red}} \)-βCl-IPy complex. This reaction can be defined as a “reductive elimination” as it involves net transfer of 2 e\(^-\) equivalents to the leaving group and (re)oxidation of the flavin. Proposals for the chemistry of such a step have been discussed earlier, e.g. in Ref. 6; some of these involve the formation of covalent adducts between the reduced flavin and βCl-IPy. This concept has been reworked and expanded in Scheme 3, taking into account newer insights and the present results. The key point is that in the π-complex between reduced flavin and iminopyruvate (\( E_{\text{red}} \)-βCl-IPy, Scheme 2), the flavin N(5) sp\(^3\) orbital that interacts with the π-orbital at C(2) of the acceptor (imino group) can be either a free pair or an N-H bond. Depending on whether the hydrogen is in either one of these orbitals, the overlap with the imino acid π-orbital can induce either (reverse) hydride transfer as in the normal reaction (step \( k_2 \), Scheme 2) or formation of the covalent adduct (C in Scheme 3). That formation of such an adduct cannot be observed by spectroscopic means might be due simply to an unfavorable equilibrium concentration of C and/or to the kinetics of the involved steps. The formation of covalent adducts between reduced flavin and carboxyls or imines has preceded both in the chemical system (30, 31) and in flavoenzymes (32, 33). Cl\(^{-}\) elimination from adduct (C) then occurs via concerted transfer of 2 e\(^-\) from the flavin to the leaving group, this being classic fragmentation as described by Grob and Schiess (34). For such fragmentations precise steric orientations of involved orbitals are necessary (in general, an antiparallel one) (34); the absence of this might be a possible reason for the absence or occurrence of β-elimination in related enzymes (4, 26, 35). In turn, the occurrence or absence of β-elimination in various enzymes might be dictated by the set-up of the active site, which determines the steric orientation of the ligand α- and β-substituents.

Of particular interest from a mechanistic point of view is the fate of the αH of βCl-d-Ala, which is found in the product pyruvate at C(3) after Cl\(^{-}\) elimination. The experimental results of the present work (Fig. 4) agree in essence with those of Walsh et al. (4, 26, 35) in that the retention of label is 25–40%. Walsh et al. (4) interpreted this as resulting from the involvement of a triprotonic base/acid such as a lysine at the active center of DAAO, this in turn resulting in an ≈3-fold dilution of the label. Scheme 3 depicts a viable and attractive alternative
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**Scheme 3. Proposed mode of elimination of Cl⁻ at the locus of reduced RgDAAO and βCl-pyruvate.** In the scheme only the middle pyrazine ring of the flavin is shown. *H denotes a labeled hydrogen. In a first step (A → B) the α-H of βCl-d-Ala is transferred to the flavin N(5) as a hydride. Note that in the resulting complex the two sp² orbitals of the reduced flavin N(5) can interact with the β-Cl-iminopyruvate α-carbon π-orbital. If the one N(5) orbital contains a hydrogen does so, this leads to a reverse hydride transfer. If the second N(5) orbital interacts as shown in B, the covalent adduct (C) is formed. In the overall process the α-H of βCl-d-Ala is first transferred to the flavin N(5) and then via the Cl-alanyl-amino group to the methylene group of the enamine intermediate (D → E). The formed iminopyruvate then undergoes release/hydrolysis to form pyruvate (F). Note the correspondence: A, Eox, βCl-d-Ala; B, I1-CT; D, I2-CT of Scheme 2.

that is based on the same concept and works in the absence of such a triprotonic functional group derived from the amino acid backbone (12, 13). It also involves a triprotonic base/acid, namely, the amino group of adduct C (Scheme 3). Accordingly, the label is first transferred from the βCl-d-Ala αC to the flavin N(5) via hydride transfer to form B. Concomitant with formation of the covalent adduct C, the N(5) label is transferred to the amino group of the adduct (Scheme 3). It remains on the same nitrogen in the enamine upon Cl⁻ elimination to form D. From this position the label is tautomerized to the C(3) position in the product iminopyruvate/pyruvate (E and F). The involvement of the triprotonic amino group in the intermediate (C) thus gives a rationale for the percentage of label incorporation (Fig. 4) and requires that there is little or no exchange of label with solvent during the elimination turnover cycle either at the reduced flavin N(5) position or at the adduct amino group. The involvement of this amino group (C) in label transfer is also in agreement with the absence of a relevant pH effect on the degree of incorporation, and spectral properties between pH 6 and 9 (see Scheme 2). An alternative mechanism in which a hydride from the reduced flavin releases Cl⁻ by direct attack at β-C of βCl-IPy in a substitution reaction is unlikely as a major process as it would lead directly to a complex Eox-IP bypassing the observed Eox-enamine complex (I2-CT).

**Elimination Starting from Reduced RgDAAO—**The finding of Cl⁻ elimination starting from reduced RgDAAO is very surprising from a mechanistic point of view. From the data of Fig. 10 and supplemental Fig. S6, it can be deduced that the intrinsic activity of reduced RgDAAO in Cl⁻ elimination must be very low. Great care was taken to ensure that RgDAAO was present exclusively in the reduced state when the reaction was started by adding βCl-d-Ala. Furthermore, supplemental Fig. S6 shows that overall elimination activity increases gradually with time concomitantly with an increase in absorbance in the 450-nm area, which reflects formation of oxidized enzyme. From this it appears that a component in the system, likely βCl-d-Ala, promotes “reoxidation.” A conceivable mechanism for this would envisage a direct interaction of the reduced flavin with βCl-d-Ala in a manner comparable with that shown in Scheme 3 and mentioned above for the Eox-βCl-Py complex. Specifically, it would involve a direct attack of the N(5)-H at the βCl-d-Ala β-carbon in which the hydride releases Cl⁻ in a substitution reaction (see supplemental Scheme S1). This would generate Eox that then enters elimination catalysis as shown in Scheme 2. It should be noted that such an elimination would constitute a slow side reaction (see Fig. 9 and supplemental Fig. S6).

**Conclusions—**After the initial reports by Walsh et al. (1, 35, 36) on the β-elimination reactions catalyzed by DAAO and the dispute on the mechanisms of dehydrogenation by flavoprotein oxidases and dehydrogenases (see for example Refs. 37–39), numerous papers have dealt with mechanisms that should circumvent the impracticability of a carbonion intermediate. A first such a proposal was discussed by the group of Massey already in 1976 (6), and it forms the basis of the present one. It takes into account the various experimental observations...
such as the dependence of Cl⁻ elimination from the presence of oxygen and in particular the retention of substrate αC⁺H label into the product pyruvate. These mechanisms also highlight the inherent capacity of enzymes to catalyze reactions that differ from those that take place during normal catalysis (promiscuity).

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