

On The Mechanism of D-Amino Acid Oxidase

STRUCTURE/LINEAR FREE ENERGY CORRELATIONS AND DEUTERIUM KINETIC ISOTOPE EFFECTS USING SUBSTITUTED PHENYLGLYCINES*

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The kinetic mechanism of the reaction of D-amino acid oxidase (EC 1.4.3.3) from *Trigonopsis variabilis* with [α - ^1H]- and [α - ^2H]phenylglycine has been determined. The pH dependence of V_{\max} is compatible with $\text{p}K_a$ values of ≈ 8.1 and >9.5 , the former of which is attributed to a base which should be deprotonated for efficient catalysis. The deuterium isotope effect on turnover is ≈ 3.9 , and the solvent isotope effect ≈ 1.6 . The reductive half-reaction is biphasic, the first, fast phase, k_2 , corresponding to substrate dehydrogenation/enzyme flavin reduction and the second to conversion/release of product. Enzyme flavin reduction consists in an approach to equilibrium involving a finite rate for k_{-2} , the reversal of k_2 , k_2 is 28.8 and 4.6 s^{-1} for [α - ^1H]- and [α - ^2H]phenylglycine, respectively, yielding a primary deuterium isotope effect ≈ 6 . The solvent deuterium isotope effect on the apparent rate of reduction for [α - ^1H]- and [α - ^2H]phenylglycine is ≈ 2.8 and ≈ 5 . The rates for k_{-2} are 4.2 and 0.9 s^{-1} for [α - ^1H]- and [α - ^2H]phenylglycine, respectively, and the corresponding isotope effect is ≈ 4.7 . The isotope effect on α -H and the solvent one thus behave multiplicatively consistent with a highly concerted process and a symmetric transition state.

The k_2 and k_{-2} values for phenylglycines carrying the *para* substituents F, Cl, Br, CH_3 , OH, NO_2 and OCH_3 have been determined. There is a linear correlation of k_2 with the substituent volume V_M and with σ^+ ; k_{-2} correlates best with σ or σ^+ while steric parameters have little influence. This is consistent with the transition state being structurally similar to the product. The Brønsted plot of ΔG^\ddagger versus ΔG_0 allows the estimation of the intrinsic ΔG_0^\ddagger as $\approx 58 \text{ kJ}\cdot\text{M}^{-1}$. From the linear free energy correlations, the relation of ΔG^\ddagger versus ΔG_0 and according to the theory of Marcus it is concluded that there is little if any development of charge in the transition state. This, together with the recently solved three-dimensional structure of D-amino acid oxidase from pig kidney (Mattevi, A., Vanoni, M.A., Todone, F., Rizzi, M., Teplyakov, A., Coda, A., Bolognesi, M., and Curti, B. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 7496–7501), argues against a carbanion mechanism in its classical formulation. Our data are compatible with transfer of a hydride from the substrate $\alpha\text{C-H}$ to the oxidized

flavin N(5) position, although, clearly, they cannot prove it.

D-Amino acid oxidase (EC 1.4.3.3, DAAO)¹ is the paradigm of flavin enzymes. It was the second flavoprotein to be uncovered, and probably it is the most studied member of this superfamily. In addition to the classical protein from mammalian kidney, recently related DAAOs have been described from various yeasts (1, 2). A common feature of all these enzymes is the dehydrogenation of D-amino acids to yield α -imino and, upon subsequent hydrolysis, α -ketoacids. The terminal redox acceptor is dioxygen. In spite of the innumerable studies, the molecular mechanism by which this enzyme brings about substrate dehydrogenation is far from being solved. Mechanistic proposals revolve around possible modes by which the substrate $\alpha\text{C-H}$ bond is being broken in the step critical for catalysis.

The most prominent proposal is the carbanion mechanism, which is characterized by initial abstraction of the α -H as H^+ leading to an intermediate in which the α -carbon carries a negative charge. Evidence in its favor has been discussed in various review articles (3, 4). So called "hydride mechanisms" in which a H^- is expelled from $\alpha\text{C-H}$ also have been discussed at various occasions but have not been proposed explicitly until most recently by Mattevi *et al.* (5). From Miura and Miyake (6) stems a proposal in which "the lone-pair electrons of the neutral amino group of the substrate are transferred to the flavin in a concerted manner with the abstraction of the α -proton." (For schematic representations of the mechanisms and structures see Denu and Fitzpatrick (7).)

An approach to investigate the molecular mechanisms of enzymes consists in the correlation of reactivities (reaction rates) with the properties of substrate substituents which influence the steric or electronic properties of the latter. This approach was advocated originally by Hammett (8) for chemical systems and was extended by Hansch and Leo (9). Klinman and co-workers (10, 11) have pioneered its use in the study of enzymatic reactions. Recently Walker and Edmondson (12) have used it to study monoamine oxidase. As early as in 1966 Neims *et al.* (13) have employed substituted phenylglycines for probing the mechanism of pkDAAO; however, the results were contradictory. In retrospect the reason for this is clear: these authors did rely on the correlations of V_{\max} data hoping to probe the reductive half-reaction. With pkDAAO the rate-lim-

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Dedicated to Prof. Dr. V. Massey, who has been one of the pioneers in the study of flavoproteins, in honor of his 70th birthday.

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¹ The abbreviations used are: DAAO, D-Amino acid oxidase; AA, amino acid; IA, imino acid; TvDAAO, D-amino acid oxidase from the yeast *Trigonopsis variabilis*; pkDAAO, D-amino acid oxidase from pig kidney; E_{ox} , DAAO oxidized form; E_{red} , DAAO reduced form; $E_{\text{red}}\sim\text{IA}$, reduced DAAO imino acid complex; $E_{\text{ox}}\sim\text{AA}$, Michaelis complex between the oxidized DAAO and substrate; LFER, linear free energy relationship.

iting step in turnover is, in general, product release (14). Later, Porter *et al.* (15) using a series of substituted phenylalanines have correlated the rate of the reversal of the dehydrogenation step of pkDAAO with the Hammett σ^+ coefficient. They interpret their positive ρ (the numerical coefficient of σ) as compatible with a carbanion mechanism. Effects of the substituents on the rate of enzyme reduction, on ΔG_0 and, by reflection of the latter on ΔG^\ddagger , did not get addressed.

It has been pointed out elsewhere (16–18) that the dehydrogenation of amino acids by DAAOs should be mechanistically related to that of α -OH acids as catalyzed, *e.g.* by lactate monooxygenase or flavocytochrome b_2 , to name only the two most prominent members of this family. This assumption has been substantiated nicely by Mattevi *et al.* (5), who have shown that the active sites of pkDAAO and flavocytochrome b_2 are mirror images resulting probably from convergent evolution. If these assumptions are correct, then the mechanistic arguments from the two subfamilies should be usable reciprocally. A major argument against a simple carbanion mechanism is, as discussed earlier (16), the finding of incorporation of the substrate α -H into the 5-deazaflavin position C(5) both in the case of pkDAAO and of α -OH acid oxidases (16, 19, 20). If a carbanion mechanism was to be operative, this would require additional steps or intermediates, since the H^+ originating from the α -C-H cannot be transferred to the flavin N(5) or C(5) concomitantly with its abstraction, unless the flavin position N(5) (or C(5) in 5-deazaflavin) would be the “abstracting base” itself, a most unlikely alternative. From this, the importance of the question about the concertedness of the reaction, as stated and studied *e.g.* by Denu and Fitzpatrick (7), becomes apparent.

During our recent studies on the catalytic mechanism of the two yeast DAAOs from *Rhodotorula gracilis* and *Trigonopsis variabilis* (21) using aliphatic D-amino acids, major differences compared with pkDAAO have emerged. Importantly, the yeast enzymes are more tolerant of variations in the chain of the amino acid; they have, overall, much higher turnover rates and have a different rate-limiting step, *e.g.* with alanine as substrate it is the reductive half-reaction, compared with product release in the case of pkDAAO (14). In view of this we have attempted to apply the concepts of linear free energy relationships (LFER) using *p*-substituted phenylglycines to probe the mechanistic questions mentioned above. The rationale behind the choice of phenylglycine is that the electronic and inductive effects of substituents on the aromatic ring should be more substantial compared with those in substituted phenylalanines as studied by Porter *et al.* (15). We have worked out the detailed kinetic mechanism for phenylglycines since we consider this to be an indispensable basis for linear free energy interpretations. Concomitantly, we have studied the primary deuterium isotope effect (on α -C-H) and the solvent deuterium isotope effect on the dehydrogenation step of phenylglycine in order to establish whether, with TvDAAO, it occurs in a concerted fashion or via intermediates. The results are interpreted in view of the recently solved three-dimensional structure of pkDAAO, the coordinates of which were made available to us prior to publication by Mattevi *et al.* (5).

EXPERIMENTAL PROCEDURES

Materials—D- and DL-phenylglycine, *p*-F-, and *p*-OH-phenylglycine derivatives were from Sigma. Phenylglyoxylic acid and its nitro-derivative were from Lancaster. DL- $[\alpha$ - 2 H]phenylglycine was prepared according to Ref. 22, and *p*-NO₂-phenylglycine according to Refs. 23 and 24. *p*-Cl-, *p*-Br-, *p*-CH₃-, and *p*-CH₃O-phenylglycine were synthesized from the corresponding substituted benzaldehydes according to Ref. 25. The purity of the synthesized compounds was checked by NMR and mass spectrometry. All other reagents were of the highest purity commercially available. DAAO from *T. variabilis* was provided by Boehringer Mannheim and was further purified according to Pollegioni *et al.*

(26). Enzyme concentration was determined using an $\epsilon_{455} = 10,800 \text{ M}^{-1} \text{ cm}^{-1}$ (27).

Polarographic Measurements—Steady state activity measurements were carried out polarographically at 25 °C in 90 mM Tris-HCl buffer, pH 8.3. All assay solutions contained 10 μM FAD and were air-saturated ($[O_2] = 0.253 \text{ mM}$). Enzyme was used in the 0.02–0.2 μM concentration range. Coenzyme and substrate solutions were freshly prepared daily. Rates were estimated from the initial velocities obtained from the linear portion of the traces using Grafit (Erithacus Software).

Rapid Reaction (Stopped-flow) Measurements—The experiments were performed at 25 °C in 50 mM Tris-HCl buffer, pH 8.3, containing 100 mM KCl. All concentrations mentioned in the context of the stopped-flow experiments are those of the reagent after mixing, *i.e.* 1:1 dilution, and refer to the D-isomer of the substrate. All the experiments were performed in a thermostatted stopped-flow spectrophotometer which has a 2-cm path length cell and which is equipped with a diode array detector (Spectroscopy Instruments, Gilching) interfaced with a MacIntosh IIcx computer using a POSMA 2.3K data acquisition program (27). The photometric response of the diode array is $\approx 80\%$, and indicated absorbance values should be corrected accordingly in order to obtain the correct values. Rapid reactions were routinely recorded in the 300–650 nm wavelength range using the normal scan mode with a scan time of 10 ms/spectra and with a resolution of 2 pixels/nm. For fast reactions ($k_{\text{obs}} > 20 \text{ s}^{-1}$) a so called “fast access” routine was used, which has an acquisition time of 1.0 ms/spectrum and a resolution of 5 nm.

For the reoxidation experiments the enzyme was reduced with a 1.5-fold excess of substrate under anaerobic conditions in the presence of 100 or 400 mM NH₄Cl and 50 mM phenylglyoxylate which can lead to formation of the reduced enzyme-ligand complex. Different $[O_2]$ in the reoxidation mixture were obtained by equilibration of the buffer solutions, containing NH₄Cl and α -ketoacid, with air (21% O₂), and with commercially available N₂/O₂ mixtures (90/10, 50/50, v/v) and pure O₂. Anaerobiosis was obtained by repeated cycles of evacuation and flushing with O₂-free argon. Prior to experiments, oxygen was scrubbed from the stopped-flow apparatus using the following procedure. The thermostating solution was flushed with N₂ at 25 °C, and the syringes were incubated with a solution of glucose and glucose oxidase (25 mM and 1 μM , respectively) for 10 h and then rinsed with deoxygenated buffer.

To assess the effect of pH on the activity of TvDAAO different buffer solutions, all containing 100 mM KCl, were used as follows: 50 mM potassium phosphate below pH 7.7, 50 mM Tris-HCl around pH 8.3, and 50 mM sodium carbonate at pH >8.9. The pH value was measured in the waste solution after the shot. Enzyme-monitored turnover data were analyzed according to the method of Gibson *et al.* (28). Program A (Dr. D. P. Ballou, University of Michigan) and KaleidaGraph (Synergy Software) were used for fittings of the kinetic traces and for data analysis and Specfit (Spectrum Software Association, Chapel Hill, NC) for deconvolution analysis. Substituent parameters (σ , σ^- , σ^+ , π , and E_s) were from Ref. 9 and V_{max} from Ref. 29. Fitting of correlations of rates with substituent parameters (Equation 4, see below) were done using Origin (Microsoft) and Statview (Abacus) and using a maximum of two variable proportionality factors.

For the study of solvent isotope effects on the reaction with D-phenylglycine, solutions were prepared by dissolving the dry substrate and buffer chemicals directly in $^2\text{H}_2\text{O}$ and by diluting a concentrated enzyme solution 16-fold in the deuterated buffer. The pD value was taken as the reading of the pH electrode plus 0.4 (30), and the pD of the solution was adjusted with DCl to the desired value.

RESULTS

Catalytic Mechanism of Yeast DAAO with D-Phenylglycine

Polarographic Measurements—The kinetic parameters V_{max} and $K_m(\text{AA})$ for D-phenylglycine as substrate were estimated with the polarographic assay at air saturation only ($[O_2] = 0.253 \text{ mM}$). The presence of the substrate L-isomer at various concentrations was found to have no effect on the measured rates, allowing the use of either the D-form or of the racemic DL-mixture. The apparent steady state parameters obtained for $[\alpha$ - ^1H]- and $[\alpha$ - ^2H]phenylglycine are reported in Table I.

Enzyme-monitored Turnover, Steady State Kinetics with $[\alpha$ - ^1H]- and $[\alpha$ - ^2H]Phenylglycine, and pH Effect—The enzyme-monitored turnover method (28) was used at different concentrations of both $[\alpha$ - ^1H]- and $[\alpha$ - ^2H]phenylglycine, and at an initial $[O_2] = 0.253 \text{ mM}$ in the range pH 5.5–9.15 by recording continuously the absorbance spectra of TvDAAO in the 350–

650 nm wavelength range. The time course of the 454 nm absorbance shows a first rapid decrease corresponding to 10–25% of the total change, which is followed by a steady state and subsequently by a final large decrease to yield the fully reduced enzyme. This behavior indicates that the overall process of (re)oxidation of the reduced DAAO forms with O₂ is faster than that involving the reductive half-reaction; it was observed at all pH values studied. At pH 8.3 and with [α -¹H]phenylglycine the enzyme is present at 78% and with [α -²H]phenylglycine at \approx 85% in the oxidized form indicating a corresponding ratio of \approx 1:4 and \approx 1:6 for the reductive and oxidative half-reactions. Significant amounts of the red anionic radical are formed, especially at low pH, when the reaction time is >100 s, resulting in an alteration of the absorption trace. In such a case the analysis was done at multiple wavelengths to minimize the spectral contribution of the radical. In spite of this, an overall uncertainty up to 15% has to be taken into account for single parameters (Table II). The Lineweaver-Burk plots of such traces show a set of converging lines at all pH values; this is in contrast to the parallel line patterns observed using D-alanine and D-valine as substrate (21). These lines intersect in the lower left quadrant, similarly to the situation reported for pkDAAO with D-phenylalanine (15). These data were analyzed assuming an ordered BiBi mechanism; plotting the slope and the intercept values of the regression lines as function of the D-phenylglycine concentration allows the estimation of K_m (AA), K_m (O₂), and K_s (AA). The corresponding steady state coefficients for [α -¹H]- and [α -²H]phenylglycine, respectively, and at pH 8.3, are reported in Table II. The estimated K_m (O₂) is very low and differs from the values previously determined with D-alanine and D-valine as substrate (0.2–0.8 mM) (21). The corresponding Dalziel coefficient for oxygen, $1/\Phi_{O_2}$, is not in agreement with the measured rate of the O₂ reaction with the reduced enzyme, for which a second-order rate constant of

$1.4 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$ was estimated (see below). As an example, at 2.5 mM [substrate] and from the Φ_{AA} and Φ_{O_2} coefficients reported in Table II, corresponding rates of 16 and 102 s⁻¹ for [α -¹H]phenylglycine and of 6 and 38 s⁻¹ for [α -²H]phenylglycine can be estimated, in good agreement with the extent of initial flavin reduction to approach the steady state phase mentioned above.

The only other substrate investigated using the enzyme-monitored turnover method at pH 8.3 was *p*-F-phenylglycine. With this substrate a set of converging lines in the Lineweaver-Burk plot is again observed, and the corresponding steady-state coefficients are reported in Table II. The values determined with this substrate are in reasonable agreement with the polarographic ones, although the measurements were performed at different oxygen and enzyme concentrations (compare Tables I and II).

The effect of pH on the k_{cat}/K_m (AA) ratio with D-phenylglycine is depicted in Fig. 1. This dependence, using the α -¹H-substrate, is consistent with the presence of two functions with apparent pK_a values of 8.1 ± 0.08 and >9.5 (the second one cannot be estimated accurately based on the present measurements), where the first must be unprotonated and the second protonated for activity. With [α -²H]phenylglycine, where, due to the low activity, only a limited number of measurements could be done at pH <7.5, only one pK_a 7.9 ± 0.1 can be estimated. The $^D V/K_m$ (AA) value, the ratio of V_{max}/K_m (AA) for [α -¹H]- and [α -²H]phenylglycine, is 6.1 ± 1.2 in the pH range 7–9.15 but does not show a definite trend.

Polarographic Measurements with *para*-Substituted Phenylglycines—The data listed in Table I show the influence of the *para* substituent on V_{max} and K_m (AA). We have attempted to use CF₃-phenylglycine as substrate; however, because of its extremely low solubility, no reliable data were obtained.

Solvent Isotope Effect on V_{max} — V_{max} values were determined with the enzyme-monitored turnover method in solvent of increasing content of ²H₂O at pH 8.3. Their dependence from the ²H₂O fraction follows a dome-shaped curve (Fig. 2). The experimental data points coincide reasonably with the theoretical curve derived from Equation 1, in which, for a good simulation, a deuterium isotope effect of 3.1 is required solely on the flavin reduction step k_2 and none on the rate of product release k_6 (see Scheme 1).

$$V_n = \frac{k_2^2 k_6}{k_2^n + k_6} = \frac{(-k_2(n-1) + k_2 n^{1/D} k_2) k_6}{(-k_2(n-1) + k_2 n^{1/D} k_2) + k_6} \quad (\text{Eq. 1})$$

where n = atom fraction of ²H₂O; k_2^n and V_n = rates at a given n ; $^D k_2$ = isotope effect on k_2 . The latter assumption is consistent with the pH independence of V_{max}/K_m (O₂) values observed by Denu and Fitzpatrick (31) with pkDAAO and D-alanine and D-serine, which was explained as resulting from low accessibility of solvent to the active site in the presence of product.

The Reductive Half-reaction with *D*-Phenylglycine—The course of the reductive half-reaction is typically biphasic as shown in Fig. 3 and similar to that found in other cases with

TABLE I
Apparent steady state kinetic parameters for the oxidation of *p*-substituted phenylglycines

Measurements at 25 °C, polarographic assay using 90 mM Tris/HCl buffer, pH 8.3; [O₂] = 0.253 mM. Numbers in italics are the isotope effects, *i.e.* the ratio of the values for the [α -¹H]- and [α -²H]- forms of phenylglycine.

<i>para</i> -Substituent	V_{max} <i>s</i> ⁻¹	K_m (A) <i>mM</i>	V_{max}/K_m <i>mM/s</i>
[α - ¹ H]-	11.8	0.5	23.6
[α - ² H]-	1.7	0.4	4.2
<i>Isotope effect</i> (¹ H/ ² H)	6.9	1.2	5.7
F-	12.5	0.2	62.5
Cl-	1.4	0.17	8.2
Br-	0.9	0.05	17.0
Me-	4.3	0.2	21.5
MeO-	3.2	0.15	21.3
HO-	13.7	0.6	22.8
NO ₂ -	2.7	0.09	31.8

TABLE II
Specific steady state coefficients for TvDAAO with [α -¹H]-, [α -²H]- and *para*-F-phenylglycine as substrate, at pH 8.3 and 25 °C

Parameter	Unit	[α - ¹ H]Phenylglycine	[α - ² H]Phenylglycine	<i>Isotope effect</i> (¹ H/ ² H) ^a	<i>p</i> -F-phenylglycine
Φ_0^{-1}	(s ⁻¹) × 10 ⁴	14.0 ± 1.5	3.6 ± 0.5	3.9	13.5
Φ_S^{-1}	(M ⁻¹ s ⁻¹) × 10 ⁴	0.65 ± 0.1	0.22 ± 0.02	3.0	8.33
$\Phi_{O_2}^{-1}$	(M ⁻¹ s ⁻¹) × 10 ⁸	40.1 ± 1.6	14.9 ± 1.5	2.7	3.3
$\Phi_{SO_2}^{-1}$	(M ⁻² s ⁻¹)	4.6 ± 1.5	2.2 ± 0.01	2.1	1.54
K_m	(mM)	2.0 ± 0.4	1.7 ± 0.3	1.2	0.16
K_{O_2}	(μ M)	37 ± 7	25 ± 5	1.5	0.41
K_{SA}	(mM)	1.35	0.58	2.3	0.22

^a For explanation of italic numbers, see legend to Table I.

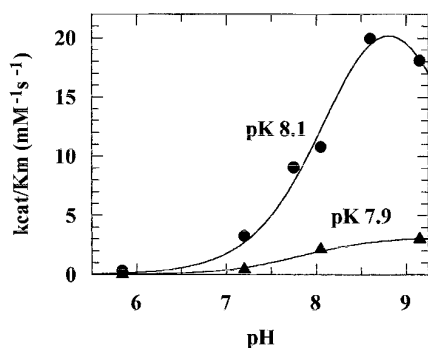


FIG. 1. pH dependence of $k_{\text{cat}}/K_m(\text{AA})$ using $[\alpha\text{-}^1\text{H}]$ phenylglycine (●) and $[\alpha\text{-}^2\text{H}]$ phenylglycine (▲). The data were obtained from steady state turnover measurements using the stopped-flow instrument and at 25 °C. See "Experimental Procedures" for details.

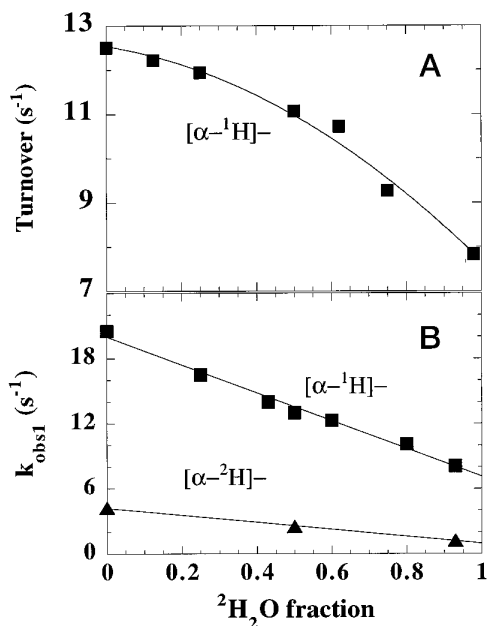
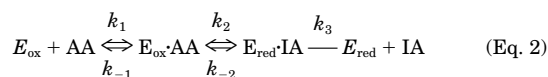
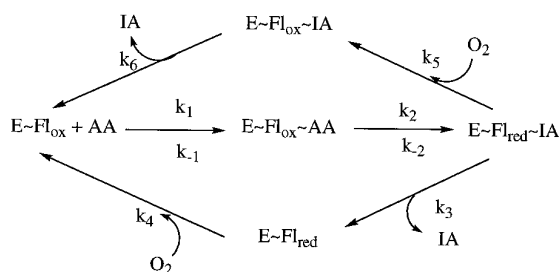


FIG. 2. Effect of the $^2\text{H}_2\text{O}$ fraction on turnover and on the reduction rate using $[\alpha\text{-}^1\text{H}]$ - and $[\alpha\text{-}^2\text{H}]$ phenylglycine. A, (■), rates of turnover with $[\alpha\text{-}^1\text{H}]$ phenylglycine measured at the fractions of $^2\text{H}_2\text{O}$ shown. — is the theoretical curve obtained using Equation 1, the constants listed in Table III, and a $k_6 = 21.5 \text{ s}^{-1}$. B, the data points are for the first, fast phase of enzyme reduction ($k_{\text{obs}1}$ monitored at 454 nm) measured with $[\alpha\text{-}^1\text{H}]$ - (■) and $[\alpha\text{-}^2\text{H}]$ phenylglycine (▲) under anaerobic conditions. For the data up to 50% $^2\text{H}_2\text{O}$ the enzyme was prepared in $^1\text{H}_2\text{O}$ -buffered solutions and the substrate in appropriate dilutions with $^2\text{H}_2\text{O}$. For the measurements in the range 50–93% $^2\text{H}_2\text{O}$ the enzyme was diluted in $^2\text{H}_2\text{O}$ buffer containing 14% $^1\text{H}_2\text{O}$, and the measurements were carried out within 120 min. Conditions: 2 mM $[\alpha\text{-}^1\text{H}]$ - and $[\alpha\text{-}^2\text{H}]$ phenylglycine at pH 8.3 and 25 °C, see "Experimental Procedures" for details.

DAAO (15, 21, 32). This is interpreted as reflecting the sequence of steps represented by Equation 2.



With phenylglycine as substrate the charge-transfer absorbance band ($\lambda > 550 \text{ nm}$) typically observed upon reaction of pKDAAO with most substrates, and which is attributed to the reduced enzyme-IA complex (33, 34), is very weak. Therefore 550 nm traces, normally employed to follow formation and decay of the $E_{\text{red}}\text{-IA}$ complex, could not be used for this purpose. For the same reason, and probably in connection with a high K_d , the titration of reduced enzyme with α -phenylglyoxylate in the presence of a large excess of ammonia did not yield



SCHEME 1. Kinetic mechanism of *Tv*DAAO with D-phenylglycine.

spectroscopically detectable species, preventing the estimation of K_d for the formation of $E_{\text{red}}\text{-IA}$ complex.

Starting with oxidized enzyme the traces at 454 nm, which reflect flavin reduction, are biphasic and were best fitted by two sequential exponentials, similar to those reported in a preceding paper for the reaction with alanine and valine (21). The observed rates for the first phase of reduction, $k_{\text{obs}1}$, exhibit saturation with increasing [phenylglycine] and a finite intercept on the ordinate as shown in Fig. 4. This is a typical case of a two-step process involving formation of an enzyme-substrate complex (steps k_1 , k_{-1} , Equation 2) followed by reversible reduction (k_2 , k_{-2}), where the y axis intercept reflects k_{-2} (35, 36). The value of k_{-2} was subtracted from $k_{\text{obs}1}$ to estimate k_2 and the apparent $K_d(\text{AA})$ using the usual double-reciprocal representation (Fig. 4, inset). The estimated kinetic parameters including the deuterium isotope effects are reported in Table III. The amplitude of the first, fast phase of enzyme reduction, $k_{\text{obs}1}$, contributes to $\approx 60\%$ of the total absorbance changes and, for $[\alpha\text{-}^1\text{H}]$ phenylglycine, the separation between $k_{\text{obs}1}$ and the ensuing phase is not very large. As a consequence the estimation of velocities as first-order rates is somewhat imprecise and the error depends on the substrate concentration. It should be noted that the apparent values of $K_d(\text{AA})$ as reflected, e.g. by the abscissa intercept in double-reciprocal plots of $k_{\text{obs}1}$ (corrected for k_{-2}) versus the concentrations of $[\alpha\text{-}^1\text{H}]$ - and $[\alpha\text{-}^2\text{H}]$ phenylglycine, yield straight lines extrapolating to a similar but not to the same value (Fig. 4, inset). This indicates a situation where true pre-equilibrium conditions ($k_{-1} \gg k_2$) are not fully satisfied as was the case also for *R. gracilis* DAAO and D-alanine (21). In order to verify this case we have carried out a simulation of the reactions with $[\alpha\text{-}^1\text{H}]$ - and $[\alpha\text{-}^2\text{H}]$ D-phenylglycine at all concentrations used and at selected concentrations of the other substrates. For obtaining a reasonable duplication of the experimental traces (cf. Fig. 3), the minimal values required for k_1 and k_{-1} were $6 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ and 40 s^{-1} for all substrates. This indicates that for $[\alpha\text{-}^1\text{H}]$ phenylglycine $k_{-1} \approx k_2$ while for the $\alpha\text{-}^2\text{H}$ -analogue the smaller value of k_2 resulting from the isotope effect leads to a true pre-equilibrium condition where $k_{-1} \gg k_2$ (35, 36). The values of the rates required for the simulation are consistent with k_{-2} being of the same order as k_2 (Table III). A particular situation concerns $[\alpha\text{-}^2\text{H}]$ D-phenylglycine where the rates of k_2 , k_{-2} , and k_3 are of the same magnitude. For this case the best simulations were obtained using values of $k_2 = 4.9$, $k_{-2} = 1.8$, and $k_3 = 2.4 \text{ s}^{-1}$, which are in good agreement with the experimentally determined values listed in Table III. A further point is the absolute rate for k_3 using $[\alpha\text{-}^1\text{H}]$ - and $[\alpha\text{-}^2\text{H}]$ D-phenylglycine, which shows an apparent isotope effect ≈ 2.2 . Extensive simulations suggest that this effect is real; although its molecular origin is unclear at present, it might be speculated that it results either from coincidence of kinetic rates or is related to steps subsequent to reduction and involving product conversion to the ketoacid and/or dissociation.

The spectral deconvolution of the primary data for the reductive half-reaction with various substrates and obtained us-

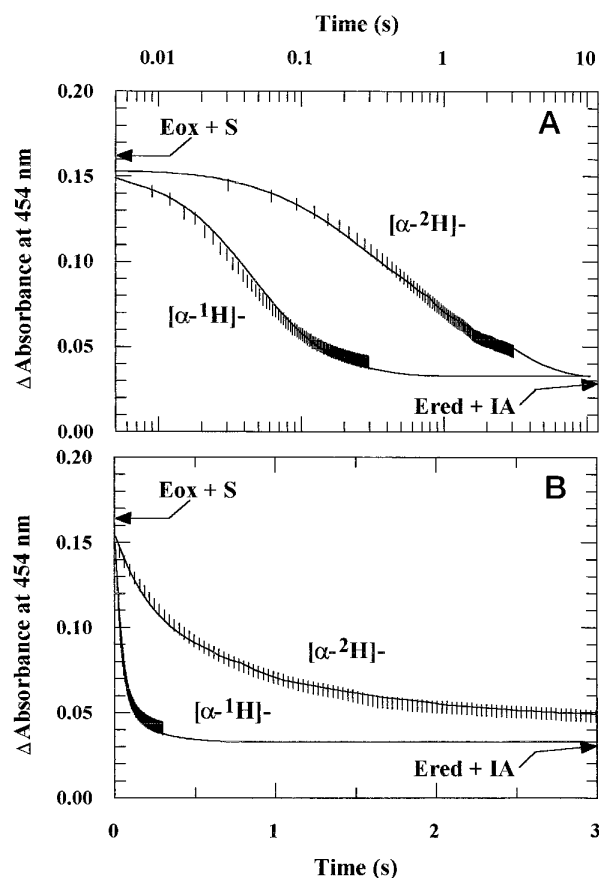


FIG. 3. Time course of anaerobic reduction of TvDAAO followed in the stopped-flow instrument. The changes in absorbance were monitored at 454 nm and are displayed on a logarithmic (A) and on a linear time scale (B). 21 μM enzyme was reacted with 1.25 mM [α - ^1H]phenylglycine and with 1.125 mM [α - ^2H]phenylglycine (final concentrations). The experimental data are indicated by the bars, and the solid traces represent the time course of A_{454} obtained by simulation using Equation 2 and the following constants: $k_1 = 60,000 \text{ M}^{-1} \text{ s}^{-1}$, $k_{-1} = 40 \text{ s}^{-1}$, $k_2 = 32$ and 4.9 s^{-1} , $k_{-2} = 5$ and 1.8 s^{-1} , $k_3 = 6$ and 2.4 s^{-1} (values for α - ^1H - and α - ^2H -labeled forms), and the (apparent) $\epsilon(E_{\text{ox}} \sim \text{AA}) = 8,640 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon(E_{\text{red}} \sim \text{IA}) = 4,350 \text{ M}^{-1} \text{ cm}^{-1}$, $\epsilon(E_{\text{red}}) = 3,350 \text{ M}^{-1} \text{ cm}^{-1}$ (as compared with the values obtained from the deconvolution analysis of 8640, 4165 and $3145 \text{ M}^{-1} \text{ cm}^{-1}$, respectively). It should be noted that ϵ should be multiplied by a factor 1.25 in order to correct for the diode array response.

ing the stopped-flow spectrophotometer equipped with a diode array detector was done with the Specfit program. In all cases three different spectral forms corresponding to the oxidized, the fully reduced, and to one intermediate, the $E_{\text{red}} \sim \text{IA}$ complex were found. These spectral species have the same extinction coefficients as those derived from single wavelength analysis of the experimental data and which were used for the simulations (see legend of Fig. 3). The value of k_2 is higher than the $1/\Phi_0$ term determined from steady state conditions. This signifies that the oxidative half-reaction is partially rate-limiting in the reaction of TvDAAO with phenylglycine, whereas with D-alanine and D-valine the reductive half-reaction is substantially smaller than the oxidative one and thus Φ_0 is $< 1/k_2$ (21). With pkDAAO and D-phenylalanine the bimolecular coefficient is significant because of the relatively large value of the reverse of the reductive half-reaction " k_{rev} " (15). The isotope effect of ≈ 6 found on k_2 is high and similar to those reported for D-alanine and D-valine (21). In the case of some substrates such as [α - ^2H]- and p - CH_3 -phenylglycine, the rate of the second phase of enzyme reduction $k_{\text{obs } 2}$ exhibits some dependence on the substrate concentration; this is consistent with k_{-2} being an important term in the reductive half-reaction and with k_{-2}

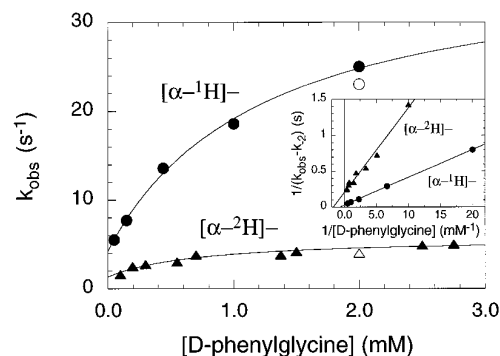


FIG. 4. Dependence of the observed rate for the first phase ($k_{\text{obs } 1}$) of the anaerobic reduction of TvDAAO on the concentration of phenylglycine. [α - ^1H]phenylglycine (\bullet) and [α - ^2H]phenylglycine (\blacktriangle). Conditions: pH 8.3, and 25°C . The inset shows the double-reciprocal plot of the same data after subtraction of k_{-2} , the y intercept of the main graph. The open symbols are the rates obtained with [α - ^1H]phenylglycine (\circ) and [α - ^2H]phenylglycine (\triangle) in $^1\text{H}_2\text{O}$ and in the experiments depicted in Fig. 2B.

being in the same order of magnitude as k_3 (see Table III). This leads to a difficult estimation of k_{-2} for these substrates. In analogy with our previous study, we assume that $k_{\text{obs } 2}$ reflects product release as such or in combination with conversion of IA to the ketoacid (21). The value of $k_{\text{obs } 2}$ ($\approx 6 \text{ s}^{-1}$, Table III) is too small to be important in turnover of the enzyme (14.0 s^{-1} , Table II); therefore, reoxidation of the reduced enzyme must result largely from reaction of O_2 with the $E_{\text{red}} \sim \text{IA}$ (or $E_{\text{red}} \sim \text{ketoacid}$) complex. The Arrhenius plot of the reductive half-reaction (k_2) of TvDAAO with phenylglycine as substrate was linear in the 15 – 35°C range, allowing to estimate an activation energy of 56 kJ/mol (data not shown).

Effects of Substituent on the Reductive Half-reaction—The time-dependent reduction of oxidized TvDAAO is biphasic with all substrates used, and the 454 nm traces are best fitted by two sequential exponential to yield values for $k_{\text{obs } 1}$ and $k_{\text{obs } 2}$, respectively. With p - CF_3 -phenylglycine no useful data could be obtained due to the low solubility of the compound. As with unsubstituted phenylglycine, with all p -substituted analogues no appreciable charge-transfer absorption was observed. In all cases the direct plot of $k_{\text{obs } 1}$ versus [p -X-phenylglycine] resulted in traces with a definite ordinate intercept similar to that observed with phenylglycine (Fig. 4), allowing the estimation of k_2 , k_{-2} , and $K_d(\text{AA})$ (cf. Equation 2, data in Table III). In all cases, the value of k_2 is higher than $1/\Phi_0$ determined under steady state conditions, confirming that the oxidative half-reaction in turnover is partially rate-limiting. With the exception of p - NO_2 -phenylglycine, the K_d values for all derivatives are within a narrow range around 0.7 mM (Table III). In contrast, the rates of enzyme reduction vary ≈ 30 -fold and those of $k_{-2} \approx 7$ -fold. Only with p -OH-phenylglycine k_{-2} is small compared with k_2 , whereas with p -Cl-phenylglycine k_{-2} and k_2 have similar absolute values. Because of the similar magnitude of k_3 and k_{-2} for many substrates, the values of k_3 reported in Table III can be considered only an estimation of this rate constant, in agreement with the values used in simulation of the experimental traces.

Solvent Isotope Effect on the Reductive Half-reaction $k_{\text{obs } 1}$ —The solvent deuterium isotope effect on the rate of enzyme reduction was estimated by varying the $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ fraction between 0 and 93% as shown in Fig. 2B. The experiments were carried out at a constant and fixed [D -phenylglycine] = 2 mM , which is close to the limit of solubility and $\gg K_m$. The experiments were carried out within 120 min in order to minimize the time of incubation of TvDAAO in deuterated solvent; indeed, the variation of the rate of reduction with the time of incuba-

TABLE III

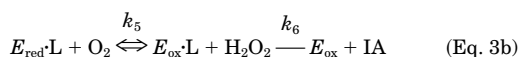
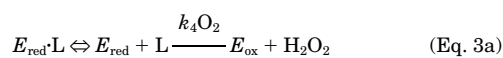
Specific rate constants of the reductive half reaction for TvDAAO with different *p*-substituted phenylglycines as substrate, at pH 8.3 and 25 °C

K_{sAA} is the abscissa extrapolation in double-reciprocal plots of k_{obs} versus substrate concentration (compare to Fig. 4). This value corresponds to the true K_d value (k_{-1}/k_1) only when a rapid equilibrium situation applies ($k_{-1} \gg k_2$), see text for details. For explanation of italic numbers, see legend to Table I.

	k_{obs1}	k_2	k_{-2}	k_2/k_{-2}	K_{sAA} ($k_{-1} + k_2/k_1$)	k_{obs2} (k_3)	K_{dAA} (k_{-1}/k_1)
	s^{-1}	s^{-1}	s^{-1}		<i>mM</i>	s^{-1}	<i>mM</i> ⁻¹
[α - ¹ H]	33.0	28.8	4.2	6.9 ± 1.0	1.2	6.0	0.7
[α - ² H]-	5.5	4.6	0.9	5.1 ± 0.9	0.7	≈2.7	0.64
Isotope effect (¹ H/ ² H)	6.0	6.3	4.7	1.35 ± 0.5	2.0	2.2	1.1
F-	36.0	32	4.0	8.0	0.23	8	
Cl-	7.0	4.0	3.0	1.3	0.4	1.7	0.38
Br-	5.3	3.3	2.0	1.7	0.11	1.4	0.18
Me	8.5	7.0	1.5	4.7	0.67	2.9	0.6
MeO-	11.0	9.6	1.4	6.7	0.4	3.2	0.28
OH-	63.0	62	1.0	62	0.7	7.5	
NO ₂ -	5.9	4.4	1.5	2.9	1	0.7	1.18

tion was negligible during the 10–15 min required for the measurements. This is somewhat surprising in that there is no apparent incorporation of deuterium in the protein in the mentioned time frame, which would affect the reduction rate. The dependence of k_{obs1} from the ¹H₂O/²H₂O fraction depicted in Fig. 2B is linear, compatible with the fission of a single exchangeable H bond during flavin reduction, and extrapolates to a solvent isotope effect of ≈2.7 (ratio of left- and right-hand side ordinate intercepts for [α -¹H]phenylglycine). This is in good agreement with the value ^D k_2 ≈3.1 estimated from turnover experiments (Fig. 2A, *cf. above*). The same type of experiment using [α -²H]phenylglycine yields a solvent isotope effect ≈4.5 (Fig. 2B). The deuterium isotope effect for the rupture of the α C-H bond in H₂O is ≈5 (Fig. 2B, ratio of left-hand ordinate intercepts). It corresponds to the ≈6 on k_{obs1} (Table III) estimated from the data of Fig. 4 at saturation. The isotope effect on k_{obs1} for the α C-H bond in ²H₂O is ≈8.5 (Fig. 2B, ratio of right-hand ordinate intercepts). Finally, the “double isotope effect” (rates of [α -¹H]phenylglycine in H₂O and [α -²H]phenylglycine in ²H₂O) is ≈24. The effect of the pH on the solvent isotope effect cannot be addressed in the present work; it is the subject of an ongoing study. The pH could have some effect on the magnitude of the isotope effects, but we consider it unlikely that it will modify them substantially and thus affect the overall conclusions. The isotope effects in question are derived from k_{obs1} which is the sum of k_2 and k_{-2} . Thus and since ^D k_2 > ^D k_{-2} , the intrinsic isotope effects on k_2 are likely to be somewhat higher, as can also be deduced from comparison of the corresponding values for k_{obs1} , k_2 , and k_{-2} obtained from [substrate] dependencies and listed in Table III.

The Oxidative Half-reaction—The (re)oxidation of reduced enzyme forms with oxygen was investigated using reduced TvDAAO in the presence of NH₄⁺ and phenylglyoxylate. It could be not studied with free E_{red} , due to its rapid conversion to the radical anion in the presence of light (21). The course of the reaction is clearly biphasic in the presence of 50 mM phenylglyoxylate and of 100 or 400 mM NH₄Cl. The experimental traces are best fitted by two exponentials and assuming parallel reactions, with the reoxidation originating from the free E_{red} , and ligand (L) complexed enzyme according to Equations 3a and 3b.



The increase (65–83%) of extent of the faster phase correlates with the [NH₄Cl] present and suggests that the concentrations

of phenylglyoxylate and NH₄Cl used are not sufficient to ensure complete E_{red} -IA formation. This situation is different compared with the reoxidation of reduced TvDAAO in the presence of pyruvate and NH₄Cl, where saturation was attained (K_d ≈2 mM (21)). The primary data obtained from these reoxidation experiments can be analyzed assuming a second-order process for both phases. The direct plot of the rates of reoxidation in the presence of 400 mM NH₄Cl yields a second-order rate $1.33 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for the first, fast phase, corresponding to k_5 (Equation 3b); it does not show indications for saturation with O₂. The rates estimated for the second, slower phase vary considerably, due to the smaller amplitude of the involved absorbance changes; they are attributed to the reoxidation of the free reduced enzyme k_4 ($8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$). In any event they are too low to be compatible with the Φ_{O_2} term determined from the steady state experiments.

Linear Free Energy Relationships of Yeast *D*-Amino Acid Oxidase

Structure-Activity Relationships of the Apparent V_{max} Using para-Substituted Phenylglycines—Correlations of V_{max} values with Hammett parameters have been attempted by Hellerman's group (13). Their correlations were strongly biphasic, comprising branches with positive and negative slopes, and could not be interpreted mechanistically. With the substrates listed in Table I correlations were attempted first with the following substituent parameters (9): electronic (σ , σ^+ , σ^-), hydrophobic (π), and steric parameters (V_M = van der Waals molar substituent volume, E_s = Taft steric parameter). A general trend is already apparent from correlations of V_{max} with single parameters in that there is a marked dependence from V_M and a dependence from σ with a ρ having a negative slope (not shown). Much better correlations were obtained using two-parameter fits and the modified Hammett equation (Equation 4) as also used by others (11, 12),

$$\log(V_{max}, k_2, \text{ or } k_{-2}) = \sigma\rho + Ax + c \quad (\text{Eq. 4})$$

where σ and ρ are the Hammett “sigma” and “rho” parameters, A is the second parameter, x a proportionality factor, and c a constant.

The results listed in Table IV indicate that V_M has a much larger effect on V_{max} compared with σ parameters. (Note that V_M parameters have larger values compared with σ ones.) The magnitude of the obtained ρ values is small, and their signs as well as that of x for V_M correlation are negative.

Structure-Activity Relationships with the Reductive Half-reaction Using para-Substituted Phenylglycines—The rate constants (k_2) determined for the reductive half-reaction with var-

ious *para*-substituted phenylglycine analogues (Table III) were correlated with electronic (σ), hydrophobic (π), and steric (E_s and V_M) parameters using Equation 4. Fits were initially attempted with single parameter correlations, but for reasonable results two parameter fits were necessary (Table V). In all cases, *p*-NO₂-phenylglycine shows an anomalous behavior and was not included in further analysis. The plots of the two-parameter ($\sigma^+ + V_M$) correlations of k_2 are shown in Fig. 5, A and B. The best correlations were obtained with σ^+ as compared with σ or σ^- , whereby, as in the case of the correlations of V_{\max} , the volume term appears to be the most important factor. In all cases the slope ρ is negative. For k_{-2} remarkably good fits are obtained already with single parameters and in particular with σ^+ (not shown, Table V). The two parameter correlations of k_{-2} with either σ^+ or σ together with V_M are of similar quality (Table V) and that with σ^+ is shown in Fig. 6.

DISCUSSION

Catalytic Mechanism of TvDAAO—As pointed out in the Introduction we have worked out the kinetic mechanism with phenylglycines as a basis for the interpretation of LFERs. The general catalytic behavior is similar to that reported previously for D-alanine and D-valine (21). There is, however, a major difference, reflected by the convergent pattern of double-reciprocal plots of steady state analysis, which is indicative of a sequential, ordered BiBi mechanism. According to Dalziel (37) the steady state of this mechanism is described by Equations 5a and 5b which base on the sequences of Scheme 1,

$$e_t/v = \Phi_0 + \Phi_{AA}/[AA] + \Phi_{O_2}/[O_2] + \Phi_{AAO_2}/[AA][O_2] \quad (\text{Eq. 5a})$$

$$\frac{e_t}{v} = \frac{k_2 + k_6}{k_2 \cdot k_6} + \frac{k_{-1} + k_2}{k_1 \cdot k_2[AA]} + \frac{k_2 + k_{-2}}{k_2 \cdot k_5[O_2]} + \frac{k_{-1} + k_{-2}}{k_1 \cdot k_2 \cdot k_5[AA][O_2]} \quad (\text{Eq. 5b})$$

where e_t = [total enzyme], v = velocity of the reaction, and $1/\Phi_0 = V_{\max}$.

TABLE IV
Correlations analysis of apparent V_{\max} values with various *p*-substituent parameters

The experimental data were obtained with the polarographic assay, using the *para* substituted D-phenylglycines listed in Table I. The parameters are from Hansch and Leo (9). σ , electronic; π , hydrophobic; Y_M , steric parameters. The values were obtained using Equation 4 for two parameters fits (R = correlation constant). Note that V_M parameters have much larger values compared to σ ones.

	Parameter					R	
	σ	σ^+	σ	π	$+V_M$		$+ \text{Constant}$
1	-1.23				-0.08	1.45	0.975
2		-0.64			-0.087	1.41	0.952
3			-1.56		-0.075	1.48	0.988
4				-0.53	-0.051	1.31	0.924

TABLE V
Correlation analysis of kinetic parameters of the reductive half-reaction of TvDAAO with *para*-substituent parameters

For the values of k_2 , k_{-2} , and the k_2/k_{-2} ratio see Table III and the legend of Table IV for further details. The values were obtained using Equation 4 for two-parameter fits. As shown in Fig. 5 and 6, the contribution of the steric factor is comparatively large (V_M range is 4–17) compared with the electronic ones (range $-1/+0.2$). (R = correlation constant, F = ratio of the regression sum of squares (explained by model) to the residual sum of squares (not explained by model) weighted by the number of data points and the degrees of freedom (higher F values correspond better fit), p = significance).

Rate constant	Parameter				$+ \text{Constant}$	R	F	p
	σ	σ^+	σ^-	V_M				
k_2	-1.255			-0.079	1.896	0.97	32.7	0.0034
		0.733		-0.0881	1.85	0.989	88.98	0.0005
			-1.39	-0.075	1.927	0.936	15.495	0.0131
k_{-2}	0.702			-0.027	0.65	0.888	7.422	0.045
		0.398		-0.0223	0.675	0.899	8.347	0.0374
			0.697	-0.0294	0.633	0.792	3.41	0.137
k_2/k_{-2}	-1.953			-0.052	1.247	0.947	17.226	0.0108
		-1.127		-0.066	1.176	0.974	37.238	0.0026
			2.082	-0.0458	1.296	0.852	5.705	0.0674

This is different from what we reported for D-alanine and D-valine (21) because the fourth term of Equation 5a is not 0 and $1/\Phi_0$ is $< k_2$, compatible with k_6 being of the same order as k_2 (Scheme 1, see also Tables II and III). Using the values of k_2 and V_{\max} for [α -¹H]- and [α -²H]phenylglycine listed in these tables, a k_6 value of 20–30 s⁻¹ can be estimated, which is close to that of k_2 . A very important difference is the reversibility of the reductive half-reaction with values of k_{-2} being dependent on the nature of the *para*-substituent and in the range 1.6 to 75% that of k_2 (see Table III).

A further difference between phenylglycine and alanine or valine as substrates concerns the ratios of the steps k_1 , k_{-1} , and k_2 , *i.e.* the question about the existence of pre-equilibrium conditions during the reductive half-reaction. The regression curves shown in Fig. 4 for [α -¹H]- and [α -²H]phenylglycine do not extrapolate to the same abscissa intercepts (see Table III for $K_{s(AA)}$ values). This is interpreted as reflecting a situation where, with α -¹H, k_{-1} is not $\gg k_2$, whereas this is the case with [α -²H]phenylglycine due to the deuterium isotope effect on k_2 . Consequently the values of $K_d (= k_{-1}/k_1)$ for [α -²H]phenylglycine can be assumed to be valid also for the α -¹H form, and k_2 can be estimated as described by Porter *et al.* (15). Note that the conspicuous isotope effect on k_2 (≈ 6) is largely conserved (*i.e.* ≈ 3.9) on turnover in agreement with the estimation of k_6 discussed above.

The rates of reoxidation estimated with the stopped-flow method are clearly not consistent with the steady state parameters Φ_{O_2} and Φ_{AAO_2} (see Equations 5a and 5b). These probably reflect reoxidation of $EF1_{\text{red}}$ via k_4 and of $EF1_{\text{red}} \sim IA$ (or $EF1_{\text{red}} \sim \text{ketoacid}$) via k_5 (Equations 3a and 3b) occurring in parallel. Indeed the simulated course of e_t/v (Fig. 3) obtained using the rate constants from rapid reaction studies (Table III) and a $k_5 = 3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ satisfactorily matches the experimental traces, indicating that this value of k_5 is close to the intrinsic one.

There are thus substantial differences between TvDAAO and pkDAAO in details of their catalytic mechanisms. These are due, however, to differences in the absolute values of single steps, the general picture remaining the same. On the other hand important similarities exist such as the coincidence of pK values of catalytic groups (8.1 and >9.5 , Fig. 1) for TvDAAO and phenylglycine compared with 8.7 and 10.7 or 8.1 and 11.5 for pkDAAO and serine or alanine, respectively (31), as expressed by the pH dependences of turnover. Also similar is the requirement of a deprotonated group with $pK \approx 8$ is for catalysis. This coincidence supports the assumption that the yeast and pig kidney enzymes operate by the same basic catalytic mechanism.

On the Concertedness of the Reductive Half-reaction—A car-

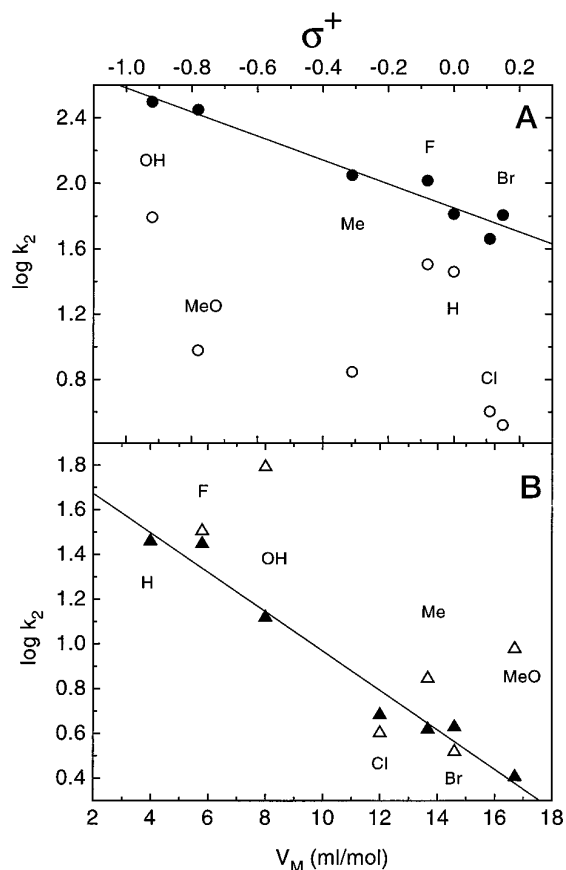


FIG. 5. Correlation of the rate constant of reduction k_2 of *TvDAAO* with substrate *para*-substituent parameters. A, uncorrected data (\circ) and data corrected for the contribution of V_M (\bullet) are plotted against the Hammett substituent parameter σ^+ . B, uncorrected data (Δ) and data corrected for the contribution of σ^+ (\blacktriangle) are plotted against the molecular volume V_M .

dinal point for the discussion of the mechanism of DAAO is whether the reductive half-reaction, which involves cleavage of the substrate α C-H and of the α N-H bonds as well as transfer of reducing equivalents to the (oxidized) flavin cofactor, proceeds in a synchronous/concerted fashion or whether distinct intermediates occur. The carbanion mechanism, for instance, is difficult to be formulated without the occurrence of intermediates. Criteria, based on which a differentiation should be possible, are *e.g.* the deuterium isotope effects observed on breaking either one of the two or both bonds involved (7, 37–39). In the case of a concerted reaction (single transition state) the increase in activation energy is due to both deuterium substitutions and will, at first approximation, be additive and thus the effect on $k_{\text{obs } 1}$ will be multiplicative. This is similar to the case of dopamine β -monooxygenase reported by Miller and Klinman (11). In contrast, in the case of the occurrence of an intermediate, the two isotope effects should behave additively or only a single one will be expressed. For *TvDAAO* the experimentally observed isotope effect on the fission of the α C-H bond (Fig. 2B) and the solvent ones compute as $2.7 \cdot 8.5 = 23$ and $5 \cdot 4.5 = 22.5$, and this compares to the experimental “double” isotope effect ≈ 24 (α C- $^1\text{H}/^2\text{H}$ and $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$, Fig. 2B). In the case of an occurrence of intermediates a value of maximally 10–12 might be expected. The reductive half-reaction is thus most likely concerted/synchronous. The linearity of the dependence of the rate of $k_{\text{obs } 1}$ from the $^2\text{H}_2\text{O}$ fraction (Fig. 2B) is compatible with fission of one exchangeable ^2H bond during dehydrogenation; a likely candidate is that of α C-N- $^2\text{H}_2/\alpha$ C-N- $^2\text{H}_3^+$. There is a discrepancy between our results and what was

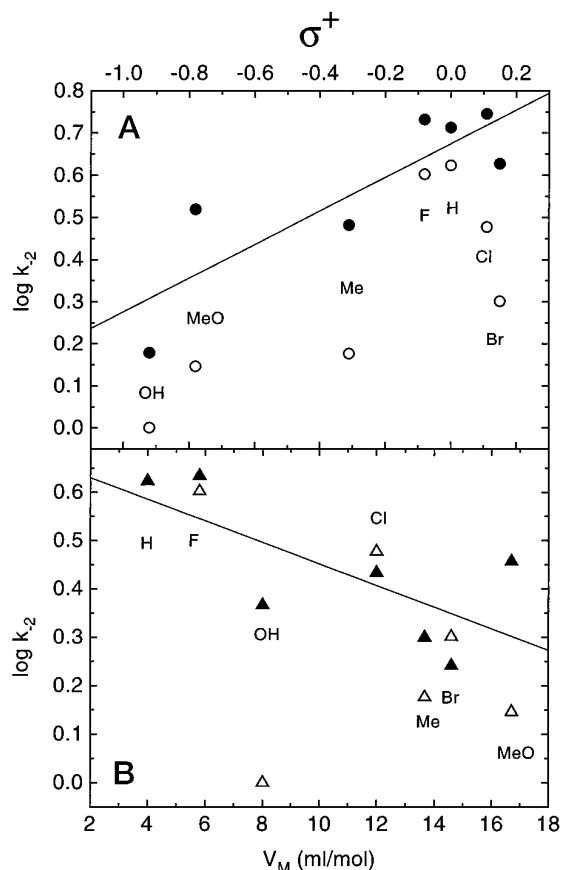


FIG. 6. Correlation of the rate constant of “reversal of reduction” k_{-2} of *TvDAAO* with substrate *para*-substituent parameters. A, uncorrected data (\circ) and data corrected for the contribution of V_M (\bullet) are plotted against the Hammett substituent parameter σ^+ . B, uncorrected data (Δ) and data corrected for the contribution of σ^+ (\blacktriangle) are plotted against the molecular volume V_M .

reported by Denu and Fitzpatrick (7) for pkDAAO and D-serine, who found no solvent isotope effect in studies of V/K_{ser} versus pH and at pH >9. In our case a solvent isotope effect was found both in turnover (Fig. 2A) and, most importantly, on the reductive half-reaction itself (step $k_{\text{obs } 1}$, Fig. 2B). Differences between pkDAAO and *TvDAAO*, in detail, *e.g.* regarding the concertedness of the reaction, are conceivable. Thus, the same authors using pkDAAO and D-alanine as substrate (40) have reported a deuterium solvent isotope effect ≈ 2.1 and ≈ 2.6 on V_{max} at pH 6.0 and 10.0 corresponding to 1 and at least 2 proton inventories, respectively. They attribute these effects to fission of an X- $^1\text{H}/^2\text{H}$ bond during product release, the rate-limiting step with pkDAAO, and to an equilibrium between two enzyme species one of these being predominant at the different pH values.

Linear Free Energy Correlations, Steric Effects, and the Active Center of *TvDAAO*—The single parameter correlation of V_{max} , k_2 , or k_{-2} values measured with the *para*-substituted phenylglycines resulted always in rather poor fits, the best statistical correlation being that of k_{-2} with the Hammett σ^+ parameter (Table V). This is in line with the results of Porter *et al.* (15), who found a good correlation of the rate of the reversal of dehydrogenation of substituted phenylalanines by pkDAAO with σ^+ . Two parameter fits, according to Equation 4, provide much better statistical correlations when the electronic parameters (σ , σ^+ , σ^-) are used in conjunction with the van der Waals volume V_M (Tables IV and V). The contribution of the steric factor is comparatively large (V_M range is 4–17) compared with the electronic ones (range $-1/+0.2$). The reasons for the re-

quirement of large steric parameters becomes evident upon inspection of the three-dimensional structure of the active site of pkDAAO complexed with benzoate (5). The aromatic ring of the latter is sandwiched between the flavin and Tyr-224, its *para*-position is close to the side chains of Leu-51 and Gln-53, and its sides are in contact with the side chains of Ile-215 and Ile-230. As can be deduced from a sequence alignment, in *Tv*DAAO groups of comparable steric requirements are in corresponding positions, namely Tyr-224 > Asp-239, Gln-53 > Leu-52, Leu-51 > Asn-50. There is no apparent open access to the exterior solvent from the substrate binding site in the pkDAAO complex with benzoate. We have modeled phenylglycine at the active center of pkDAAO replacing benzoate (5), the position of both $-\text{COO}^-$ being constant. This shows that the aromatic ring of phenylglycine points toward the solvent and is close to it. The active site of *Tv*DAAO must be more flexible or open to solvent since it is able to accommodate a large volume variation at the *p*-position of phenylglycine. This is also in agreement with the finding of little change in the dissociation constants $K_d (=k_{-1}/k_1)$ of unsubstituted phenylglycine and of all *p*-substituted analogues, with the exception of *p*-NO₂ (Table III). In view of the startling similarities of the active centers of pkDAAO and α -OH-acid oxidases uncovered by Mattevi *et al.* (5), it is tempting to assume that the active center of *Tv*DAAO is more similar to that of the latter, where the substrate chain extends toward and is in contact with the solvent. In fact, using lactate oxidase or lactate monooxygenase and a series of mandelates carrying the same substituents as the phenylglycines used in this work, a similar independence of K_d from the substituent was observed.²

During dehydrogenation, and irrespective of the involved mechanism, a conversion of the substrate pyramidal sp^3 α -carbon center into a planar sp^2 product occurs, and this requires substantial movement of the three αC substituents. Importantly, in the intermediate transition state a product-like configuration with sp^2 character will be encountered. Based on this one would predict large steric effects on k_2 and much less pronounced ones, if any at all, for the "back reaction" k_{-2} . The experimental findings correspond fully to this expectation (Tables IV and V, Figs. 5 and 6). In other words, "steric work" (movement of the substrate substituents and/or active side residues) is necessary for the interconversion of substrate and transition state, not (or little) for that of the transition state into product. The three-dimensional features of DAAO also provide a rationale for the deviant behavior of *p*-NO₂-phenylglycine. The $-\text{NO}_2$ group is the largest in the series, it is highly polar, and specific interactions can be expected to occur, *e.g.* with Asp-239 or Asn-50. It is worth noting that also in the case of monoamine oxidase B data obtained with NO₂-substituted substrates do not fit in LFER correlations (12).

Linear Free Energy Correlations, Electronic Effects, and the Reaction Mechanism of Dehydrogenation—While the validity of Hammett type correlations for the understanding of organic reaction mechanisms is undisputed (41), their interpretations in the case of enzymatic mechanisms is much more difficult due to the uncertainties arising from interactions with the protein. Positive precedents for the use of the LFER approach with flavin enzymes are bacterial luciferase (42) and monoamine oxidase by Walker and Edmondson (12).

We subdivide our analysis of the correlations with electronic parameters into that of k_2 , that of k_{-2} (yielding the ΔG^\ddagger , the activation energy for the interconversion of the substrate and imino acid via the transition state), and that of the ratio of k_2/k_{-2} (yielding ΔG_0 the apparent free energy of the reaction,

excluding binding steps). ΔG^\ddagger for k_2 can be affected either by substituent induced changes of the energies of the substrates ground state and/or by changes of transition state energies (*cf.* Fig. 8). The variation of K_d values for formation of the Michaelis complex within the series of substrates used is from 18.0 to 20.9 $\text{kJ}\cdot\text{M}^{-1}$ and thus negligibly small, *i.e.* $\Delta G_0 \approx \text{constant}$ ($\approx 19 \text{ kJ}\cdot\text{M}^{-1}$). In the case of *p*-*X*-phenylglycines/*p*-*X*-phenyliminoglyoxylates, substituent induced differences in ground state free energies are likely to be much larger on the side of products due to the through conjugation. For this reason we have normalized the ground state free energy levels of bound substrates as shown on the left-hand side of Fig. 8. From the plots of Fig. 5, it is evident that for the σ^+ correlation of k_2 ρ is negative and has a small value of -0.733 . Taken at face value, this would suggest that *p*-substituents exert little electronic effects, and, if at all, a partial positive charge develops in the transition state.

For the σ^+ (or σ) correlation of k_{-2} , on the other hand, ρ is positive (0.398 and 0.702, respectively), *i.e.* opposite as compared with the same correlation with k_2 . The similarity of the absolute values of ρ for the σ^+ correlations of k_2 and k_{-2} satisfies the principle of microreversibility. For completing the picture, differences in ground state energies of products (complexed to the enzyme) have to be considered. Redox potentials are not available for the series of *p*-substituted phenylglycines used in this work. But simple chemical considerations suggest that, qualitatively, the *p*-*X* substituent will exert a strong(er) effect on the ground state free energy of products. Electron withdrawing substituents should increase it, and donating ones have the opposite effect (41). In the representation of Fig. 8 dissociation of products from E_{red} has not been taken into account. On one hand it was not addressed experimentally; on the other hand only steps up to $E_{\text{red}} \sim \text{IA}$ (Equation 2) are of relevance for the intended correlations. The ground state energy levels of bound products (ΔG_0) have thus been estimated from the ratio $k_2/k_{-2} \approx K_e$, the internal redox equilibrium of the (de)hydrogenation reaction.

The assessment of the effect of ground state energy levels on ΔG^\ddagger is of importance for the discussion of substituent effects as has been discussed also by Miller and Klinman (11). In this context, Marcus (43) has derived a relation (Equation 6) which describes the effect of ΔG_0 on ΔG^\ddagger for reactions involving the transfer of H^+ ,

$$\alpha = (1 + \Delta G_0/4\Delta G_0^\ddagger)/2 \quad (\text{Eq. 6})$$

where α is the Brønsted slope which can be obtained from the plot of ΔG^\ddagger against ΔG_0 (43). We have attempted to apply this correlation using the ΔG_0 and ΔG^\ddagger values discussed above. The variation of ΔG^\ddagger values for all *p*-substituted phenylglycines is small; it ranges from 54.9 for Cl^- to 50.1 $\text{kJ}\cdot\text{M}^{-1}$ for OH-phenylglycine. From a Brønsted plot of our data (the rate constants were corrected for the steric effects, thus they reflect only electronic substituent effects), as shown in Fig. 7, we can estimate α as ≈ 0.63 . According to Marcus (43) "the Brønsted slope α is expected to be 0.5, when $\Delta G_0 = 0$." The experimentally determined and the theoretically expected values of α are thus very close. It is difficult to judge whether any of the reasons for deviation from the value of 0.5 discussed by Marcus (43), and which imply asymmetries in the reaction profiles, would apply for our case of an enzymatic reaction. It should be kept in mind, however, that in the present case the profile of the reaction is most probably quite symmetrical. The second pertinent quote from Marcus (43) states that "in the region of $\alpha = 0.5$, σ and ρ then depend only on variations in ΔG_0 ." This questions indirectly the validity of Hammett type correlations done in the absence of information on ΔG_0 .

² S. Ghisla, K. Yorita, and V. Massey, unpublished results.

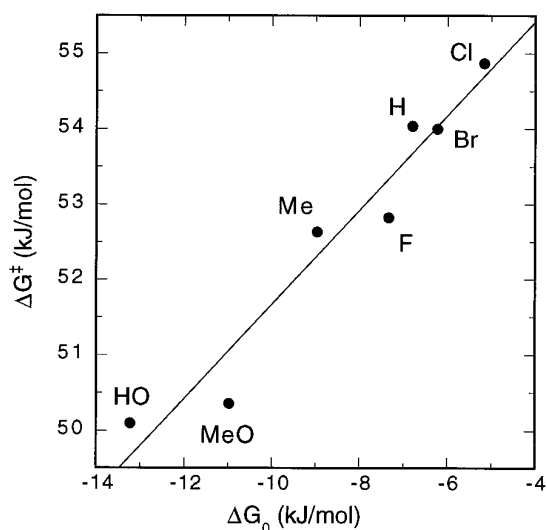


FIG. 7. Brønsted plot of ΔG^\ddagger versus ΔG_0 . The curve (—) is the theoretical dependence obtained using Equation 6. 56 kJ/mol is the energy of activation for $[\alpha\text{-}^1\text{H}]$ phenylglycine, *i.e.* ΔG^\ddagger . The ΔG^\ddagger for the other substrates was calculated relatively to the latter value using the Arrhenius equation and the k_2 values reported in Table III after correction for steric effects. ΔG_0 values were calculated from the k_2/k_{-2} ratio, again after correction for steric effects. All these corrections were carried out based on the best correlation equation, *i.e.* that with σ^+ and V_M .

In our case, and in view of these considerations, we think it is safe to conclude that in the dehydrogenation of phenylglycines catalyzed by TvDAAO and as described by Equation 2, there is probably little if any development of (positive) charge in the transition state. This minimal interpretation is also in agreement with the finding of the best correlation of k_2 and k_{-2} with σ^+ , the Hammett parameter for positively charged transient species. The validity of our reasoning is supported by the analysis of the correlations with V_M (*i.e.* upon correction for the electronic effects, Figs. 5B and 6B). In this case a Brønsted correlation with $\alpha \approx 1.2$ is obtained (not shown), suggesting that the steric requirements of the substituents have an important effect on the energy level of the transition state. From the Brønsted plot of Fig. 7 the intrinsic (*i.e.* substituent independent or that determined only by electronic effects) reaction barrier of the dehydrogenation reaction ΔG_0^\ddagger (intrinsic) can be estimated as $\approx 58 \text{ kJ}\cdot\text{M}^{-1}$.

Mechanistic Conclusions—Mattevi *et al.* (5) have proposed convincingly that the active site of flavocytochrome b_2 (and thus of the α -OH-acid hydroxylase family) can be described as the mirror image of that of DAAO and that the localization of benzoate in DAAO coincides with that of pyruvate in flavocytochrome b_2 . We have done an analysis similar to the present one using L-lactate oxidase and L-lactate monooxygenase and a series of *p*-substituted mandelic acids.² In these cases the rate of flavin reduction correlates with the substrate substituent Hammett σ value, and there is no necessity for correction for steric parameters. Therefore the assumption mentioned in the introduction that the two enzyme classes operate by a similar mechanism is reasonable. Our data are compatible with any mechanism in which little or no charge is developing in the transition state. The energetic profile of the reaction should be symmetrical (Fig. 8), the fission of involved bonds occurring synchronously. This does not exclude "carbanion mechanisms," as long as intermediates do not carry substantial negative charge at the substrate α C. This is the case in the formulation by Miura and Miyake (6) but not in that of Lederer (18) for flavocytochrome b_2 or that for lactate monooxygenase (44). Clearly, the interpretation of the present data in terms of a

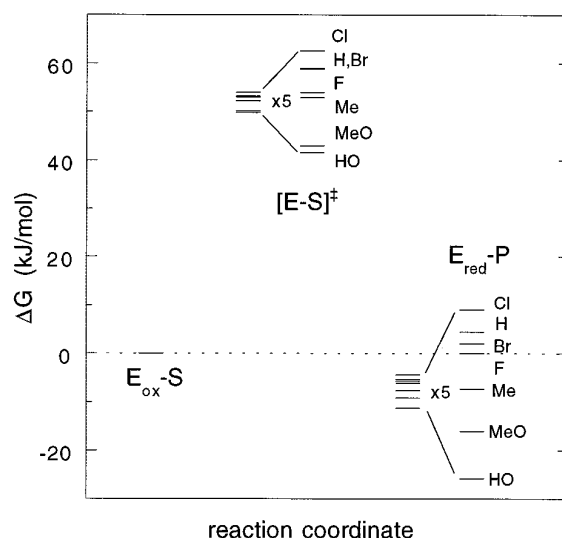
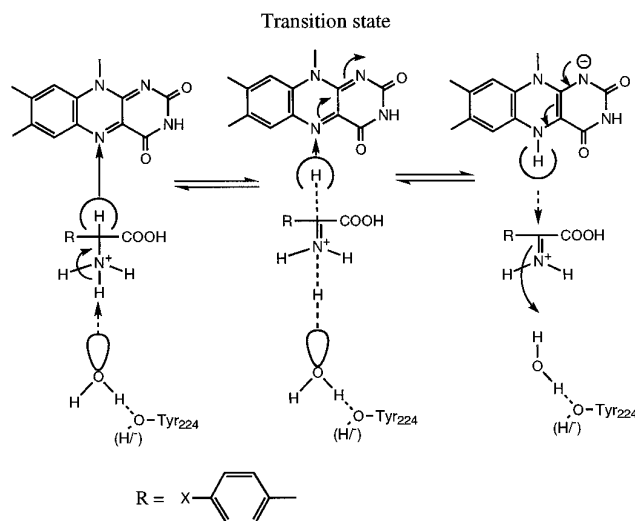


FIG. 8. Energy diagram of the reductive half-reaction of TvDAAO and dependence from the *para*-substituent of phenylglycines. See text and legend to Fig. 7 for further details.



SCHEME 2. Postulated catalytic mechanism of D-amino acid oxidase. The numbering of Tyr-224 refers to the three-dimensional structure of pkDAAO (5).

hydride transfer mechanism, as done independently by Mattevi *et al.* (5), is much more convincing. The base at the active center of pkDAAO which might function in H^+ abstraction is Tyr-224, possibly coupled to a H_2O molecule. Removal of the Tyr-OH group by mutagenesis (Tyr-224-Phe) leads to a protein with substantial activity (45). The same holds for the other tyrosine residue at the active center (Tyr-228-Phe, (45)). The cases of these two mutants would lead to the seemingly paradox situation in which just no functional group capable of acid/base catalysis is left at the active center of active DAAO, while the enzyme is still active! In a hydride transfer mechanism and for the Tyr-224-Phe mutant, during the conversion $\alpha\text{NH}_3^+ \Rightarrow \alpha=\text{NH}_2 + \text{H}^+$ (or $\alpha\text{NH}_2 \Rightarrow \alpha=\text{NH} + \text{H}^+$), H^+ just would dissociate into solvent. With normal DAAO removal of the same H^+ would be promoted by H_2O linked to Tyr-224 and as shown in Scheme 2. This role would be exerted by a histidine within the family of α -OH-acid dehydrogenases (*e.g.* His-373 in cytochrome b_2 (18)). The difference in functional groups (Tyr *versus* His) between the two types of "dehydrogenases" would reflect an evolutive adaptation to the different $\text{p}K_a$ values of the substrate $\alpha\text{-XH}$ group ($X = \text{O}$ or NH).

We are aware that the conclusions arising from the present work are apparently difficult to reconcile with the carbanion or with other mechanisms proposed previously by others and also by ourselves. An option worth reconsidering might be the occurrence of different mechanisms depending on the type of substrate (16). Finally it is impossible to discuss in the present context all the arguments concerning these mechanisms which were put forward over the years in several dozens of papers; this will have to be done in *proprii loci*.

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