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On the Mechanism of D-amino Acid Oxidase: Structure/Reactivity Studies using *p*-substituted Phenylglycines

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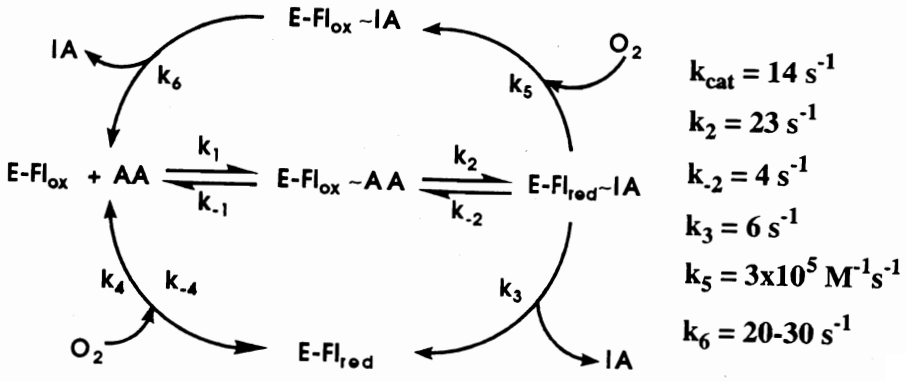
INTRODUCTION

In spite of innumerable studies, the molecular mechanism by which D-amino acid oxidase (EC 1.4.3.3, DAAO) catalyzes the dehydrogenation of D-amino acids is far from being understood. Various proposals deal with the modes by which the substrate α-C-H bond is being broken. The carbanion mechanism is initiated by abstraction of the α-H as H^+ and leads to an intermediate in which the substrate α-C carries a negative charge. In the "hydride mechanism" a H^- is expulsed from αC-H. Combinations of these mechanisms also has been proposed (1).

We have addressed the question about the mechanism of DAAO and of mechanistically related enzymes such as the α-OH-acid "dehydrogenases" using the DAAO from the yeast *Trigonopsis variabilis*. Hereby we have attempted Linear Free Energy Relationships (LFER) of the reaction rates obtained using a series of *p*-substituted phenylglycines, and the classical parameters for the influence of steric or electronic properties of the *p*-substituents (2) were used. These analyses have been combined with measurements of primary and solvent isotope effect to assess the concertedness of the dehydrogenation reaction. The results are interpreted in view of the recently solved 3D-structure of pkDAAO, the coordinates of which were made available to us prior to publication (3).

METHODS

DAAO from *T. variabilis* was provided by Boehringer Mannheim; enzyme concentration was determined using an ε_{455}= 10,800 $M^{-1}cm^{-1}$. Steady state activity measurements were carried out polarographically at 25°C in 90 mM Tris•HCl buffer, pH 8.3 at air-saturation. Rapid reaction (stopped-flow) measurements were performed at 25°C in 50 mM Tris•HCl buffer, pH 8.3, containing 100 mM KCl. For activity measurements at different pH vales buffer KCl, were: 50 mM potassium phosphate at pH <7.7, 50 mM Tris-HCl ≈ pH 8 and 50 mM Na_2CO_3 at pH >8.9.



SCHEME 1. Kinetic mechanism of the reaction of TvDAAO with D-phenylglycine and principal rate constants.

Substituent parameters (σ, σ^-,σ^+, π and E_S) were used as listed in Hansch and Leo (2) and V_M values were taken from Bondi (4). For the study of solvent isotope effects, solutions were prepared by dissolving the dry substrate and buffer chemicals directly in ^2H_2O and by diluting a concentrated enzyme solution 16-fold in the deuterated buffer. pH value in ^2H_2O is the pHmeter reading + 0.5.

RESULTS

Catalytic Mechanism of Yeast DAAO with D-phenylglycine

The kinetic mechanism of TvDAAO was investigated using the polarographic assay at air saturation with various *para*-D-phenylglycine and using the enzyme monitored turnover method with [α-^{1}H]- and [α-^{2}H]-phenylglycine) With all the substrates tested (and at all pH values) TvDAAO shows an ordered BiBi mechanism (upper loop of Scheme 1). This is different from what we reported for D-alanine and D-valine (5) because k_6 is of the same order as k_2 (Scheme 1). The value of k_3 (6 s^{-1}) is too small to be important in turnover (14.0 s^{-1}), therefore reoxidation of the reduced enzyme must result largely from reaction of O_2 with the E_{red}~IA complex. The reversal of the reductive half-reaction is also dependent on the nature of the *para*-substituent and k_{-2} is in the range 1.6 to 75 % that of k_2. The rates of enzyme reduction vary ≈30-fold and those of k_{-2} ≈7-fold, while for all derivatives the K_d values are within a narrow range around 0.7 mM. The Arrhenius plot of the rate of

reduction (k_2) of TvDAAO with phenylglycine is linear in the 15-35°C range ($\Delta G \approx$ 56 kJ/mol).

The k_{cat}/K_{mAA} ratio with phenylglycine is pH dependent, corresponding to apparent pK_a values ≈ 7.9 and ≈ 8.0 for the [α-^{1}H]- and [α-^{2}H]-forms. These pK values are probably close to the intrinsic ones, as previously reported for pkDAAO and D-serine as substrate (6).

Solvent Isotope Effects on V_{max} and on the Rate of Enzyme Reduction

V_{max} values show a curved dependence from the ^2H_2O content (inset, Fig. 1). The experimental data points coincide reasonably with a theoretical curve in which a deuterium isotope effect of 3.1 only on the flavin reduction step k_2, is required for a good fit and none on the rate of product release k_6 (Scheme 1).

The solvent deuterium isotope effect on the rate of enzyme reduction (k_2) was estimated by varying the ^1H_2O/ ^2H_2O fraction between 0 and 93% as shown in Fig. 1. Surprisingly there is no apparent incorporation of deuterium in the protein during the time required for the measurements (<20 min), which would affect the reduction rate. The dependence of k_2 from the ^1H_2O/ ^2H_2O fraction is linear, compatible with the fission of a single exchangeable H-bond during flavin reduction, a likely candidate being the αC-N-^2H. It extrapolates to a solvent isotope effect of ≈ 3, in good agreement with the value $^Dk_2 \approx 3.1$ estimated from turnover experiments (Table I). The deuterium isotope effect for the rupture of the αC-H bond in H_2O is ≈ 5 The isotope effect on the rate of rupture of the αC-H bond (k_2) in ^2H_2O is ≈ 8, and the "double isotope effect" found for the ratio of the rates of [α-^{1}H]-phenylglycine in H_2O and [α-^{2}H]-phenylglycine in ^2H_2O is ≈ 25 (Table I). It is important to note that the solvent deuterium isotope effect and that for rupture of the αC-H bond clearly behave multiplicatively as opposed to additively. These isotope effects might contain contributions from "pK shifts" which can occur in deuterated solvents, and which are not accounted for by the "pH correction factor of 0.5" (7) used in the present work for the measurements in ^2H_2O. The magnitude of such an unaccounted effect is unlikely to be the sole cause of the observed deuterium isotope effect (a comprehensive study of the pH dependence of the isotope effects is in preparation). In spite of this reservation we consider the observation of multiplicative deuterium effects as compatible with a synchronous/concerted mode of rupture of the two bonds of substrate during dehydrogenation. This contrasts with a mechanism in which intermediate occurs, and proceeds stepwise.

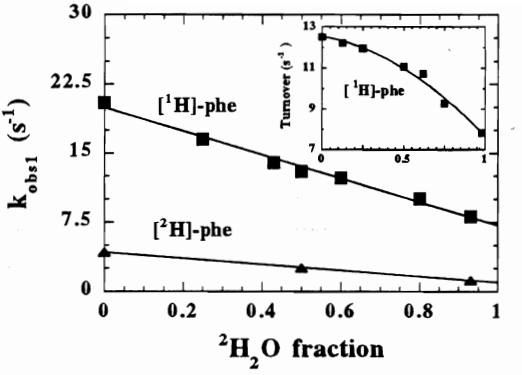


FIG. 1. Effect of the ^2H_2O fraction on the reduction rate and on turnover (inset) of TvDAAO using [α-^{1}H]- and [α-^{2}H]-phenylglycine. Conditions: the first, fast phase of enzyme reduction (k_{obs1}) was measured with 4 mM [α-^{1}H]- and [α-^{2}H]-phenylglycine, at pH 8.3 and 25°C. The rates were estimated from the absorbance changes at 454 nm.

TABLE I. Summary of deuterium isotope effects with *T. variabilis* DAAO.

Substrate phenylglycine	Parameter:	in H_2O	in ^2H_2O*	*solvent isotope effect*
[α-^{1}H]-	V_{max}	14	8	*1.6*
[α-^{2}H]-	V_{max}	3.6		
isotope effect		*3.9*		
[α-^{1}H]-	k_2	21 (23.3)	7	*3*
[α-^{1}H]-	k_2	4.0 (4.1)	0.8	*3*
isotope effect		*5.2*	*8.5*	
[α-^{1}H]-	k_{-2}	7		
[α-^{1}H]-	k_{-2}	1.8		
isotope effect		*4*		
[α-^{1}H]-	k_2/k_{-2}	3.3		
[α-^{1}H]-	k_2/k_{-2}	2.3		
isotope effect		*1.4*		

Values in brackets for k_2 are extrapolated from the dependence of k_{obs1} from [S].
* Values obtained at [phenylglycine] = 4 mM

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. Two parameter fits,

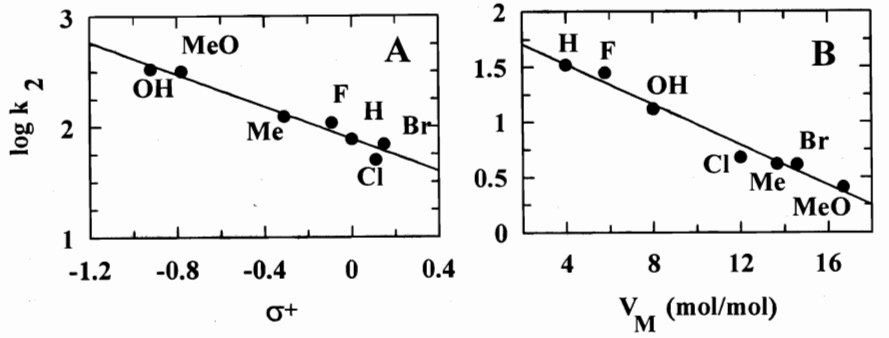


FIG. 2. Correlation of the rate constant of reduction k_2 of TvDAAO with *para*-substituted phenylglycines and using Hammett substituent parameters σ^+ (panel A) and V_M (panel B). Note that the data points shown are corrected for the other parameter.

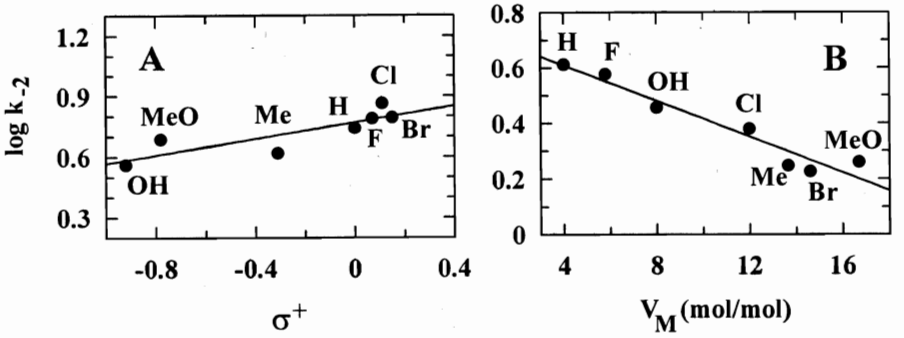


FIG. 3. Correlation of the rate constant of "reversal of reduction" k_2 of TvDAAO with *para*-substituted phenylglycines and using the Hammett substituent parameter σ^+ (panel A) and V_M (panel B). Note that the data points shown are corrected for the other parameter.

according to eq. 2, provide significantly better statistical correlations (the electronic parameters (σ, σ^+,σ^-), the van der Waals volume (V_M) or the Taft steric parameter (E_S) were used):

$$\log V_{max} = \sigma\rho + Ax + c \qquad \text{(eq. 2)}$$

where $\sigma\rho$ are the Hammett "sigma" and "rho" parameters, "A" is the second parameter, "x" a proportionality factor and "c" a constant.

In the correlation analysis of V_{max}, V_M has a much larger effect compared to σ and both ρ and the slope of V_M are negative. The plots of the two-parameter (σ^+ + V_M) correlations of k_2 and k_{-2} are shown in Fig. 2 and 3. With k_2 the slope of ρ has a small negative value, suggesting that *p*-substituents exert little electronic effects.

Thus, if at all, a partial positive charge develops in the transition state. For k_{-2} remarkably good fits are obtained already with single parameters, and in particular with σ^+. In this case it is immediately apparent that ρ is again very small and positive, opposite as compared to that with k_2. There is little effect of V_M on the quality of the fits, implying that the substituent volume as such has a minor effect on k_{-2} (Fig.3). This indicates that "steric work" is necessary for the interconversion of substrate into the transition state, however, not for that of transition state into product. This is compatible with conversion of the substrate pyramidal sp3 α-carbon center into a quasi planar transition state and into a planar sp2 product during dehydrogenation.

The reasons for the requirement of large steric parameters also becomes evident upon inspection of the 3D-structure of the active site of pkDAAO complexed with benzoate (2). The aromatic ring of the latter is sandwiched between the flavin and Y224, its *para*-position is close to the side chains of L51 and Q53, and its sides are covered by the side chains of I215 and I230 (a comparable steric requirements is found at corresponding positions in TvDAAO by comparison of the primary sequences). Probably, the active site of TvDAAO is more flexible or open to solvent since it is able to accommodate a large volume variation at the *p*-position of phenylglycine, as deduced also by the little change in the binding constants K_d (= k_{-1}/k_1) of all *p*-substituted analogs.

The reaction profile of the reductive half-reaction derives from the analysis of our correlations of k_2, k_{-2} (yielding the $\Delta G^{\ddagger}$, the activation energy, for the conversion of the substrate or iminoacid into the transition state) and k_2/k_{-2} ratio (ΔG_0, the apparent free energy of the reaction, excluding binding steps). With the series of substrates used a similar value of K_d's for formation of the Michaelis complex was determined, indeed $\Delta G_0 \approx$ constant (19.2 $kJ \cdot M^{-1}$). The ground state free energy levels of free substrates have been normalized because the differences induced by the substituent can safely be assumed to be much larger on the side of products due to the through conjugation.

In our case, the average $\Delta G^{\ddagger}$ value including all *p*-substituted phenylglycines is small, it ranges from 56 for Cl- to 49.2 $kJ \cdot M^{-1}$ for OH-phenylglycine. From a Brønsted plot of our data, *i.e.* from the plot of $\Delta G^{\ddagger}$ against ΔG_0 (data not shown), we can estimate the slope α as 0.6. According to Marcus theory (8) and his equation:

$$\alpha \approx (1 + \Delta G_0 / 4\Delta G^{\ddagger})/2 \qquad \text{(eq. 3)}$$

"the Brønsted slope α is expected to be 0.5, when $\Delta G_0 = 0$", in good agreement with our experimental results. According to Marcus when $\alpha \approx 0.5$, then σ and ρ depend only on variations in ΔG_0. It is thus likely, that in our case ρ depends only

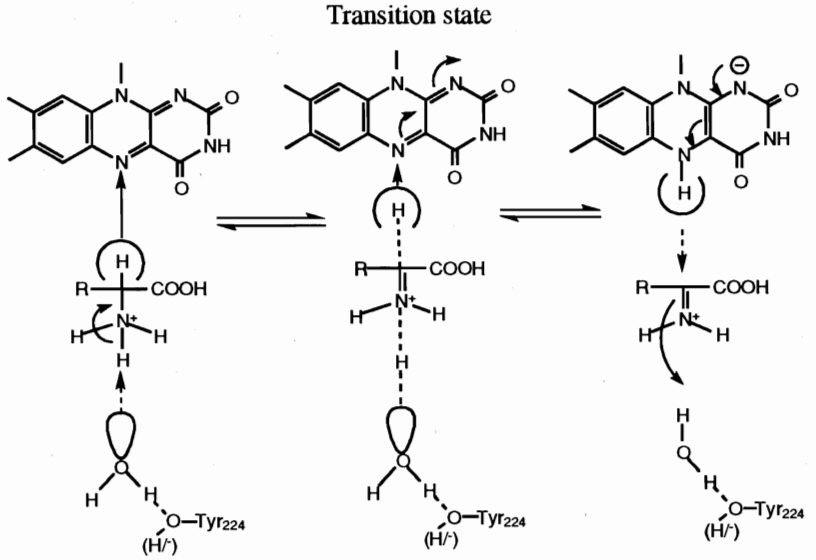


FIG. 4. Proposed hydride mechanism for DAAO catalysis.

from ΔG_0. The deviation of α from 0.5 is compatible with development of very little positive charge at the reaction center during the reaction. The validity of our reasoning is supported by the analysis of the correlation of k_2 with V_M (upon correction for the electronic effect). This has a high value of $\alpha \approx 1.7$ compatible with the steric requirements of the substituent having an important effect on $\Delta G^{\ddagger}$. From the Brønsted plot the intrinsic (*i.e.* substituent independent) reaction barrier of the dehydrogenation reaction $\Delta G^{\ddagger}$ (intrinsic) can be estimated as 59 kJ•M^{-1}, in good agreement with the Arrhenius activation energy of 56 kJ•M^{-1} determined for the dehydrogenation of normal phenylglycine.

Mechanistic conclusions

The following can de deduced from the present data:

a) Little or no charge is developed in the transition state. If charge is developed, it is likely to be positive (correlations of k_2 and k_{-2} with σ^+).
b) The reaction profile is highly symmetrical, the fission of involved bonds occurs synchronously/concertedly.
c) Steric movement of the substrate α-substituent(s) are important for the formation of the transition state.

It should be made clear, that these conclusions do not prove any mechanism. They do, however, restrict the frame in which a given mechanism has to be placed. Thus a "carbanion mechanisms" in which bond fission occurs concertedly and induces no charge on the substrate α-center is viable. This would, however, require a redefinition of the term "carbanion mechanisms" since no anionic species is being formed.

A mechanistic formulation based solely on the present data and in terms of a hydride transfer mechanism does not pose particular problems if one discerns from the long held concept, that the flavin is not a good hydride acceptor. If one includes the recently solved structure of pig kidney DAAO reported by Mattevi *et al.*(2) the conclusion becomes compelling. The only base which might function in H^+ abstraction at the active center of pkDAAO is Tyr224. Mutation of Tyr224 to phenylalanine produces an enzyme with appreciable activity (9). This means that dehydrogenation in DAAO can proceed without the aid of any base. In a hydride mechanism such as that formulated in Fig. 4 a H^+ just leaves the α-NH_3^+ group during formation of α=NH_2^+. This might undoubtedly be favoured by the pK of α-NH_3^+ which is probably around 8. In the case of the α-OH of α-hydroxy-acid "dehydrogenase" such as flavocytochrome b_2, lactate oxidase, lactate monooxygenase, or glycollate oxidase, a histidine appears to be necessary for efficient abstraction of H^+ from α-OH (10,11).

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