Substrate analogues as probes of the catalytic mechanism of L-mandelate dehydrogenase from *Rhodotorula graminis*

Otto SMÉKAL,* Graeme A. REID† and Stephen K. CHAPMAN*‡

Edinburgh Centre for Molecular Recognition, *Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland, U.K. and †Institute of Cell and Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JR, Scotland, U.K.

A detailed kinetic analysis of the oxidation of mono-substituted mandelates catalysed by L-(+)-mandelate dehydrogenase (L-MDH) from *Rhodotorula graminis* has been carried out to elucidate the role of the substrate in the catalytic mechanism. Values of K_m and $k_{cat.}$ (25 °C, pH 7.5) were determined for mandelate and eight substrate analogues. Values of the activation parameters, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (determined over the range 5–37 °C), for mandelate and all substrate analogues were compensatory resulting in similar low values for free energies of activation $\Delta G^{\ddagger}$ (approx. 60 kJ·mol^{-1} at 298.15 K) in all cases. A kinetic-isotope-effect value of 1.1 ± 0.1 was observed using D,L-[2-^{2}H]mandelate as substrate and was invariant over the temperature range studied. The logarithm of $k_{cat.}$ values for the enzymic oxidation of mandelate and all substrate analogues (except 4-hydroxymandelate) showed good correlation with Taft's dual substituent constant $\bar{\sigma}$ (where $\bar{\sigma} = \sigma_I + 0.64\sigma_R^+$) and gave a positive reaction constant value, ρ, of 0.36 ± 0.07. This linear free-energy relationship was verified by analysing the data using isokinetic methods. These findings support the hypothesis that the enzyme-catalysed reaction proceeds via the same transition state for each substrate and indicates that this transition state is relatively non-polar but has an electron-rich centre at the α-carbon position.

INTRODUCTION

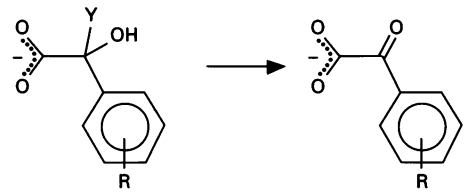
Although 2-hydroxyacids are relatively stable towards oxidation by dioxygen, stereospecific flavoenzymes that catalyse the oxidation of such compounds are widely distributed in nature. One such enzyme is the L-(+)-mandelate dehydrogenase (L-MDH), from the yeast *Rhodotorula graminis*, which catalyses the oxidation of L-(+)-mandelate to phenylglyoxylate [1,2]. L-MDH belongs to a family of enzymes, the flavocytochromes b_2, which includes the L-lactate dehydrogenases (L-LDH) from the yeasts *Saccharomyces cerevisiae* and *Hansenula anomala* [3]. In a detailed comparison of L-MDH from *R. graminis* and L-LDH from *S. cerevisiae*, it was evident that the two enzymes have mutually exclusive substrates [4]. It was also shown, by kinetic isotope effect (KIE) measurements, that the transition states in the two enzymes must be different [4]. These results raised several questions about the catalytic mechanism in L-MDH. To gain further information on this mechanism we have investigated the reactivity of L-MDH with a range of ring-substituted substrate analogues which varied in the position (*meta* or *para*) and electron-withdrawing/donating power of the ring substituent. We report here a detailed kinetic study on the oxidation of mandelate and eight substituted mandelates to the corresponding phenylglyoxalates by L-MDH as shown in Scheme 1. The mandelates used have been numerically designated as shown and are referred to using the appropriated number throughout.

We also describe the resulting structure–activity correlations and demonstrate the application of extended forms of linear free-energy relationships as an aid to understanding and verifying these correlations.

MATERIALS AND METHODS

Isolation of enzyme

L-MDH from *R. graminis* (strain KGX39) was prepared as previously described [4]. Enzyme concentrations were measured using previously published molar absorption coefficients [5].



Scheme 1 Oxidation of mandelate and eight substituted derivatives to their corresponding phenylglyoxalates

Name	Number	R	Y
L(+)-Mandelate	**1a**	H	H
D,L-Mandelate	**1b**	H	H
DL-[2-^{2}H]Mandelate	**1c**	H	^{2}H
4-Chloro-D,L-mandelate	**2**	4-Cl	H
4-Bromo-D,L-mandelate	**3**	4-Br	H
4-Fluoro-D,L-mandelate	**4**	4-F	H
4-Methyl-D,L-mandelate	**5**	4-Me	H
3-Methoxy-D,L-mandelate	**6**	3-OMe	H
4-Methoxy-D,L-mandelate	**7**	4-OMe	H
3-Hydroxy-D,L-mandelate	**8**	3-OH	H
4-Hydroxy-D,L-mandelate	**9**	4-OH	H

Substrate analogues

L-Mandelate, D,L-mandelate and all substituted D,L-mandelates were obtained from Aldrich and were > 99% pure. All of these substrates were used without further purification. D,L-Mandelate deuterated at the C-2 position, D,L-2-[^{2}H]mandelate, was prepared by a previously reported procedure [4]. The purity of this deuterated mandelate was checked by t.l.c. The isotopic purity was ascertained by ^{1}H n.m.r. spectroscopy and mass spectrometry as previously reported [4].

Kinetic measurements

Kinetic experiments were carried out between 5 °C and 37 °C (with an accuracy of ±0.1 °C) in Tris/HCl buffer at pH 7.5 and

‡ To whom correspondence should be addressed.

I 0.10. The buffer concentration was 10 mM in HCl with *I* adjusted to 0.10 M by addition of NaCl.

Steady-state kinetic measurements involving the enzymic and non-enzymic (uncatalysed) oxidation of 2-hydroxyacid substrates were carried out using Beckman DU62 or Pye–Unicam SP8-400 spectrophotometers. Reaction temperatures were maintained with the use of a Grant LTD6 circulatory water bath. Ferricyanide was used as electron acceptor and the reaction was monitored at 420 nm using an absorption coefficient of 1010 $M^{-1} \cdot cm^{-1}$ [6].

Steady-state parameters

Reaction rates, *v*, were measured from initial slopes (up to 10% product formation) of absorbance changes with time. With some substrate analogues the uncatalysed reaction made a significant contribution to the reaction rate as indicated by eqn. (1).

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_{cat.}} E + P \qquad (1)$$

$$k_{uncat.} \downarrow$$

$$P$$

Where $k_{uncat.}$ is the rate constant for 'spontaneous' or non-enzymic oxidation of the substrate, and $k_{cat.}$ and K_m are the classical Michaelis–Menten constants. The value of $k_{uncat.}$ is easily measured in the absence of enzyme and this value can then be subtracted from the observed rate constant, $k_{obs.}$, in the presence of enzyme to give the initial rate, v_0, for the enzyme catalysed reaction as shown in eqn. (2).

$$v_0 = (k_{obs.} - k_{uncat.})[S_0] \qquad (2)$$

Observed rate constants were measured at various concentrations of the purified enzyme (5×10^{-9} to 1×10^{-7} M). The relationship between initial rates, corrected for the uncatalysed reaction and substrate concentration, showed typical saturation behaviour [7]. Values of K_m and $k_{cat.}$ were determined using a non-linear regression analysis [8]. Such parameters were obtained from at least four separate reactions, with reproducibility better than ±10% (mean values are quoted).

Kinetic isotope effects for L-MDH were determined using D,L-[2-^{1}H]mandelate and D,L-[2-^{2}H]mandelate as substrates.

Activation parameters and isokinetic methods

Activation parameters; $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta G^{\ddagger}$ were determined from the dependencies of the kinetic parameters $k_{cat.}$, K_m, and $k_{cat.}/K_m$ on the temperature *T* (K) using a linear form of the Eyring equation [9]. For enzyme-catalysed oxidations, kinetic runs were performed from between 5 °C and 37 °C in approx. 5 °C steps. Non-enzymic oxidations were studied from 15 °C to 80 °C. The enhancement of the enzyme-catalysed reaction with respect to the uncatalysed reaction was calculated according to eqn (3).

$$\delta\Delta G^{\ddagger} = -RT\ln(k_{cat.}/k_{uncat.}) \qquad (3)$$

Verification of isokinetic relationships was carried out by the method of Exner [10].

RESULTS AND DISCUSSION

Kinetic isotope effects

L-MDH from *R. graminis* belongs to a family of enzymes known as the flavocytochromes b_2 which includes L-LDH from *Saccharomyces cerevisiae* [3,4]. In L-LDH it has been shown from deuterium kinetic isotope effects, ^{2}H-KIEs, that the rate-determining step of the reaction is abstraction of the C-2 hydrogen of lactate as a proton [3,5]. However, it has been reported recently that there is no observable ^{2}H-KIE for the oxidation of mandelate by L-MDH and this was interpreted as clear evidence that the transition states in the two enzymes were different [4]. This result is confirmed in the present study as direct kinetic measurements on D,L-mandelate and its deuterated analogue D,L-[2-^{2}H]mandelate give rise to a negligible KIE value of 1.1±0.1 at 25 °C. This KIE value varied little with temperature ranging from 1.03±0.15 at 5 °C to 1.24±0.19 at 35 °C, i.e. the same within experimental error. Thus we can completely rule out abstraction of the C-2 hydrogen of L-mandelate making any significant contribution to the rate-determining step of the reaction. This result raises questions about the nature of the transition state and the contribution of the substrate in the oxidation of mandelate catalysed by L-MDH.

Steady-state kinetics

For mandelate and all eight substituted mandelates the enzyme catalysed reaction was found to accurately follow first-order kinetics with respect to substrate over at least three to four half-lives. Initial rates for the enzymic oxidation of all substrates studied were measured over a range of substrate concentrations (0.01–20 mM). Saturation kinetic behaviour was observed throughout [11], with no evidence for substrate or product inhibition. Resulting $k_{cat.}$ and K_m values are listed in Table 1.

Activation parameters

An enzyme, in common with all catalysts, interacts with its substrate to form a transition state which must be of lower free energy than that for the uncatalysed reaction. To understand the nature of the transition state one must therefore have an estimate of the free energy of its formation. The activation energy for the formation of the transition state which rate limits the enzyme-catalysed reaction, can be determined by analysing the dependence of rate on temperature. Thus, for each substrate, the variation of $k_{cat.}$ and K_m values with temperature was investigated. The data were fitted to a linear form of the Eyring equation (Figure 1) and resulting activation parameters for $k_{cat.}$

Table 1 Steady-state kinetic parameters for the reaction of L-MDH with mandelate and substituted mandelates

All experiments were carried out at 25 °C in Tris/HCl buffer, pH 7.5 (*I* 0.10). Ferricyanide (1 mM) was used as electron acceptor. Values of $k_{cat.}$ are expressed in mol electrons transferred/mol enzyme per second. Kinetic parameters at 25 °C were estimated by non-linear least-squares analysis of their values from the Eyring equation.

Substrate	R	$k_{cat.}$ (at 25 °C) (s^{-1})	K_m (at 25 °C) (mM)	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
1a	H	114±6	0.24±0.04	4.7×10^5
1b	H	94±5	0.35±0.05	2.6×10^5
1c	H	93±5	0.74±0.11	1.3×10^5
2	4-Cl	116±6	0.38±0.06	3.0×10^5
3	4-Br	108±5	0.26±0.04	4.1×10^5
4	4-F	98±5	0.16±0.02	6.0×10^5
5	4-Me	80±4	0.17±0.03	4.8×10^5
6	3-OMe	106±5	0.12±0.02	8.7×10^5
7	4-OMe	68±3	0.12±0.02	5.7×10^5
8	3-OH	108±5	0.15±0.02	7.4×10^5
9	4-OH	146±7	0.08±0.01	1.9×10^6

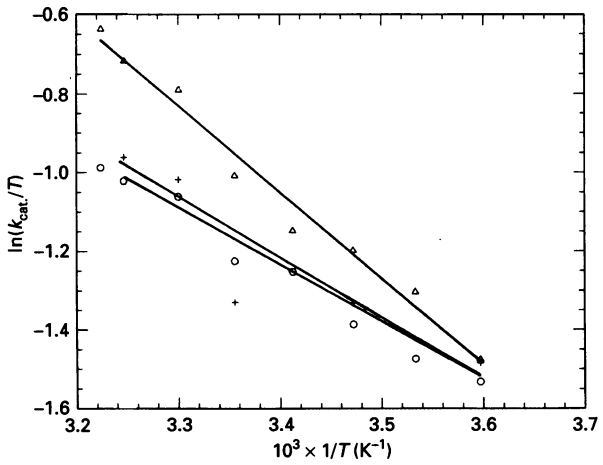


Figure 1 Eyring plots of $\ln(k_{cat.}/T)$ versus $1/T$ for the oxidation of various mandelates by *R. graminis* L-MDH

Key to symbols: △, L-(+)-mandelate; +, D,L-mandelate; ○, D,L-[2-^{2}H]mandelate.

Table 2 Values of $k_{cat.}$ and corresponding activation parameters for the reaction of L-MDH with mandelate and substituted mandelates

All experiments were carried out in Tris/HCl buffer, pH 7.5 (/ 0.10). Ferricyanide (1 mM) was used as electron acceptor. The temperature range was 5–37 °C. Values of $\Delta G^{\ddagger}$ (25 °C) were determined from the relation of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ at 25 °C.

Substrate	R	$k_{cat.}$ (at 25 °C) (s^{-1})	$\Delta G^{\ddagger}$ (at 25 °C) ($kJ \cdot mol^{-1}$)	$\Delta H^{\ddagger}$ ($kJ \cdot mol^{-1}$)	$\Delta S^{\ddagger}$ ($J \cdot mol^{-1} \cdot K^{-1}$)
1a	H	114±6	61.3	18.1±1.0	−145±10
1b	H	94±5	61.8	11.8±0.7	−168±13
1c	H	93±5	61.8	12.1±2.7	−167±50
2	4-Cl	116±6	61.3	8.6±2.7	−177±8
3	4-Br	108±5	61.4	10.1±4.5	−172±10
4	4-F	98±5	61.6	13.2±2.4	−162±4
5	4-Me	80±4	62.2	18.5±1.1	−147±11
6	3-OMe	106±5	61.5	11.9±4.6	−166±8
7	4-OMe	68±3	62.5	23.8±1.1	−130±7
8	3-OH	108±5	61.4	10.6±2.2	−170±5
9	4-OH	146±7	60.6	21.5±1.3	−131±9

are listed in Table 2. For $k_{cat.}$ measurements, the substituent effects on $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ values compensate for one another to give rise to an almost constant, low, free energy of activation $\Delta G^{\ddagger}_{298}$ of 60 $kJ \cdot mol^{-1}$. This is in contrast with the much larger free energy for the non-enzymic or uncatalysed reaction which was found to be approx. 100 $kJ \cdot mol^{-1}$. The rate enhancement for the enzyme-catalysed oxidation of mandelate compared with that for the reaction in the absence of enzyme ($k_{cat.}/k_{uncat.}$) is estimated to be about 1×10^8, and by using eqn. 3 the value of $\delta\Delta G^{\ddagger}$ was found to be 44.1 $kJ \cdot mol^{-1}$.

The relatively low enthalpies of activation and more negative entropies of activation (Table 2) are typical for an intramolecular enzyme–substrate reaction. The near constancy of the free energies of activation, $\Delta G^{\ddagger}$, for the oxidation of all of the substituted mandelates by L-MDH is strongly indicative that the same mechanism applies in all cases. However, much more informative data on the importance of structural features of the substituted mandelates have been obtained from analysis of the isokinetic relationships.

Isokinetic relationships

If a given reaction series follows a common mechanism with the same rate-determining step then there should be a quantitative relationship between enthalpy and entropy of activation, i.e. the so called isokinetic relationship or compensation law [10]. From the activation parameters in Table 2 there appears to be just such a compensation between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, consistent with an isokinetic relationship in the data for L-MDH. We have used the methods recommended by Exner [10,12] to analyse our data in order to confirm such a relationship. Using eqn. (4) excellent correlations were obtained when log $k_{cat.}$ (i+5 °C) was plotted against log $k_{cat.}$(5 °C) (Figure 2).

$$\log k_{cat.}(i+5\ °C) = a + b \cdot \log k_{cat.}(5\ °C) \quad (4)$$

(where i = temperatures from 0 to 35 °C rising in 5 °C steps).

Additional verification for the isokinetic relationships was obtained from a statistical analysis of the Arrhenius plots for each of the substituted mandelates (Figures 3 and 4). The Arrhenius plots were compared to allow the identification of a common intersection point at a particular temperature, the 'isokinetic temperature', β (Figures 3 and 4). From the statistical analysis [13,14] of the Arrhenius plots for $k_{cat.}$ (Figure 3) it can be seen that substrates **1–8** show a common intersection at an isokinetic temperature, $\beta = 332 \pm 11$ K, with only substrate **9** (4-hydroxy-D,L-mandelate) deviating from this. In fact 4-hydroxy-D,L-mandelate is the most efficient substrate with L-MDH, having a $k_{cat.}/K_m$ value some 4-fold greater than D,L-mandelate itself (Table 1).

In the case of the statistical analysis of the Arrhenius plots for the apparent K_m values, $(K_m)_{app.}$, (Figures 4a and 4b) three substrates (**7**, **8** and **9**) deviate from the others, which give an isokinetic temperature, β, of 271±14 K. It is important to note here that the substrate which deviates from the isokinetic relationships for both $k_{cat.}$ and K_m is the 4-hydroxy-D,L-mandelate and one obvious explanation for this deviation is that 4-hydroxymandelate has the potential to form an additional hydrogen bond not available to the parent substrate. The formation of such a hydrogen bond might be expected to lead to changes in $k_{cat.}$ and K_m and thus cause a deviation from the isokinetic relationship. In the case of the K_m analysis only, the 4-methoxy and 3-hydroxy derivatives also deviate from the isokinetic relationship. This would suggest that these substituents form additional interactions upon enzyme–substrate-complex formation but not on transition-state formation as they still correlate well in the $k_{cat.}$ analysis.

Structure–activity correlations

The oxidation of various substituted mandelates by L-MDH can be analysed using a Hammett-type approach. The Hammett treatment aims to correlate the effect of a *meta*- or *para*-ring substituent on the rate of reaction. Thus, attempts were made to correlate $k_{cat.}$ values with the classical Hammett relation [15], (eqn. 5) and these gave reasonable agreement (Table 3, Fit no. 1–6).

$$\log(k_{cat.})_R = \log(k_{cat.})_H + \sigma\rho \quad (5)$$

(The Hammett equation is a linear free-energy relationship where: σ values are numbers which sum up the total electronic effects, resonance plus inductive, of a group R attached to the ring; and ρ is the reaction constant. Reactions with a positive ρ are helped by electron withdrawing groups and reactions with a negative ρ are helped by electron-donating groups.) Excluding 4-

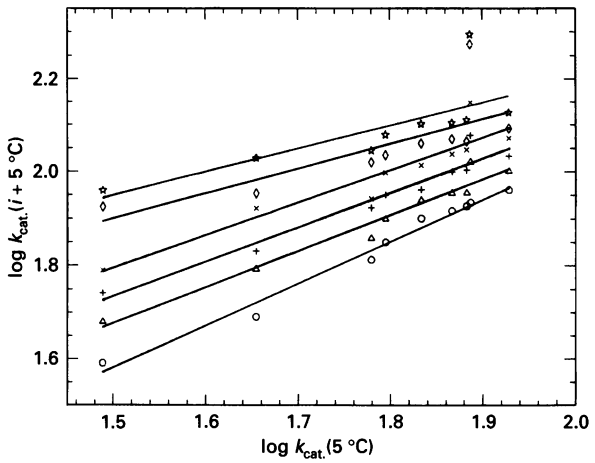


Figure 2 Isokinetic correlations using Exner's method [12], plots of ln $k_{cat.}(T_{i+5\ °C})$ versus ln $k_{cat.}(5\ °C)$ for the L-MDH catalysed oxidation of mandelates 1b,2–8

$T_{i+5\ °C}$ is > 5 °C, where i = 5 (○), 10 (△), 15 (+), 20 (×), 25 (◇), and 30 °C (☆).

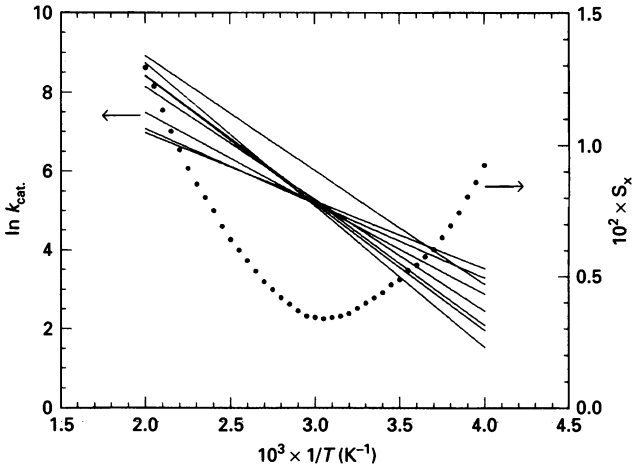


Figure 3 Statistical analysis of Arrhenius plots for $k_{cat.}$ for the oxidation of mandelates 1b,2–9 catalysed by L-MDH

Solid lines are experimental Arrhenius plot. The dotted line represents the S.D., S_x, when the Arrhenius plots are constrained to pass through a common point at the isokinetic temperature, β, at the minimum of the S_x plot. The arrow pointing to the left indicates the axis for the solid lines and the arrow pointing to the right indicates the axis for the dotted line.

hydroxymandelate, the $k_{cat.}$ values for all substrates correlated fairly well with the standard type of Hammett parameters; σ, σ_m, σ_I, σ_p [16,17], (Table 3, Fit no. 1–6). All fits resulted in a reasonable magnitude for the reaction constant, ρ. The ρ values from Table 3 are all relatively low (< 0.5) indicating that the ring substituents have only a small demand on the electron density at the C-2 carbon of the transition state. This is consistent with a fairly electron-rich C-2 carbon in the transition state for the reaction.

To gain further insight into the separate contributions of inductive and resonance effects we analysed the data in terms of dual substituent parameters [17,18] (Table 3, Fit no. 7–12). This

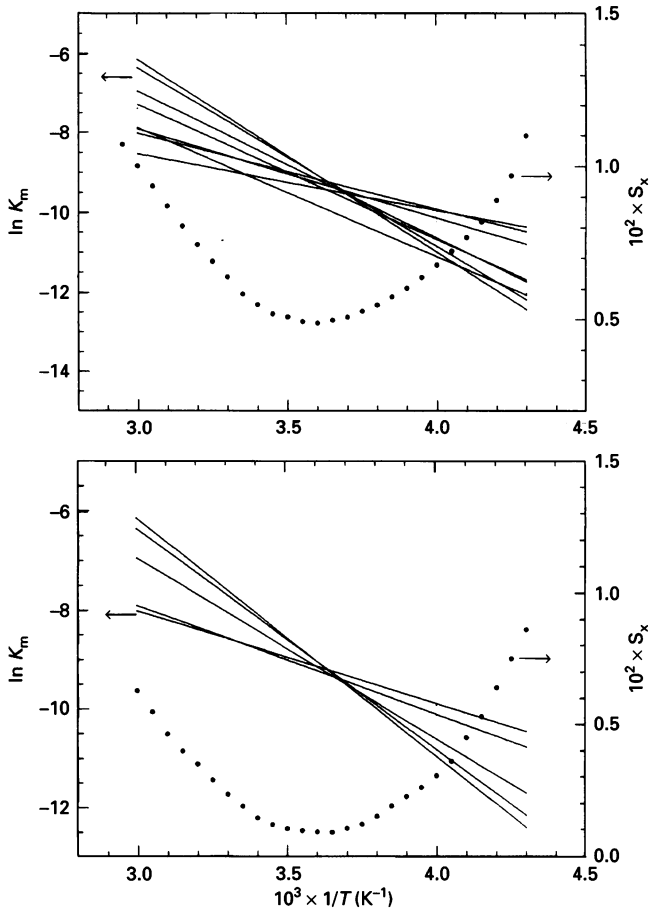


Figure 4 Statistical analysis of Arrhenius plots for K_m for the oxidation of mandelates 1b,2–9 (a), and 1b,2–6 (b), catalysed by L-MDH

Solid and dotted lines are as defined in Figure 3.

Table 3 Substituent-dependence of $k_{cat.}$ (25 °C) for the oxidation of mandelates 1b,2–8 by L-MDH

Fit number	Substituent constant*	Hammett ρ values†	Statistical F-test‡	Correlation quality ψ§
1	σ	0.45 (±0.08)	408	0.10
2	σ_m, σ_p^+	0.24 (±0.05)	190	0.11
3	σ_m, σ_p^-	0.48 (±0.23)	90	0.27
4	$\sigma_I\sigma_p$	0.44 (±0.09)	410	0.10
5	σ_m, σ_R^-	0.09 (±0.29)	20	0.49
6	σ_m, σ_R^+	0.10 (±0.16)	70	0.29
7	$\sigma_m+0.2(\sigma_p^+-\sigma_p)$	0.38 (±0.08)	201	0.11
8	$\sigma_m+1.2(\sigma_p^+-\sigma_p)$	0.22 (±0.08)	210	0.11
9	$\sigma_m+0.2(\sigma_p^--\sigma_p)$	0.45 (±0.10)	405	0.10
10	$\sigma_m+1.2(\sigma_p^--\sigma_p)$	0.44 (±0.27)	61	0.30
11	$\sigma_I+0.2\sigma_R^+$	0.25 (±0.13)	105	0.27
12	$\sigma_I+0.6\sigma_R^+$	0.38 (±0.03)	593	0.09
13	$\sigma_I+1.0\sigma_R^+$	0.50 (±0.24)	405	0.27
14‖	$\sigma_I+0.64\ (\pm0.09)\sigma_R^+$	0.36 (±0.07)	622	0.08

* From [16, 17].
† Errors in parentheses were estimated from the 95% confidence limit by the t-test.
‡ 95% confidence limit.
§ From [12].
‖ Parameters for this fit obtained were obtained by multiple linear regression.

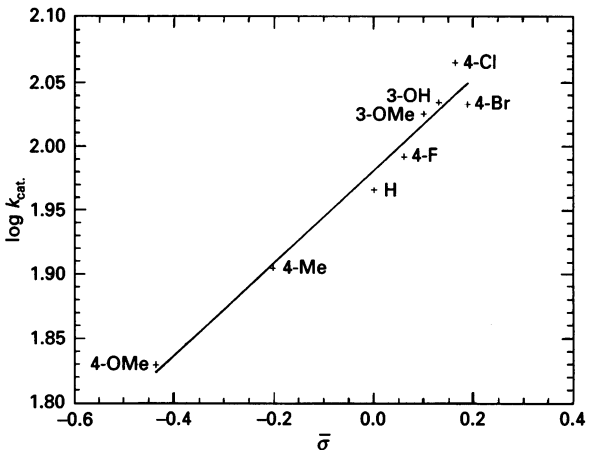


Figure 5 Hammett plot

The linear relationship between log $k_{cat.}$ for the oxidation of substituted mandelates by L-MDH, and the optimum combination of Hammett–Taft parameters [$\bar{\sigma} = (\sigma_I + 0.64\sigma_R^+)$].

correlation is given by Taft's dual substituent parameters equation (eqn. 6) [15,19].

$$\log(k_{cat.})_R = \log(k_{cat.})H + \rho(\sigma_I + \lambda\sigma_R^+) \quad (6)$$

Where $(k_{cat.})_R = k_{cat.}$ for substituted mandelate R; $(k_{cat.})_H = k_{cat.}$ for mandelate (**1b**); σ_I is the value for the inductive effect; and σ_R^+ the value for the resonance effect. Values of ρ and λ were calculated from a series of determinations on the eight different substituted mandelates by multiple linear regression with a S.D., S_{xy}, of 1.36×10^{-2}; a correlation coefficient, r, of 0.9969; an F-test, of 622 [20]; and Exner's correlation quality [21], ϕ, of 0.08. Values were found to be: $\rho = 0.36$ (± 0.07); and $\lambda = 0.64$ (± 0.09). The value of λ gives an estimate of the resonance demand on the C-2 carbon of mandelate by the β-aryl ring. The best-fit value of λ, which was found to be 0.64, indicates that the relative importance of the resonance effect is only around two-thirds that of the inductive effect.

The reason for the resonance effect being less important can be viewed in terms of the structure of the transition state. If the transition state in L-MDH involves the formation of a carbanion-type species at the C-2 position, as is believed to occur in L-LDH, then maximum resonance stabilization would be achieved if the C-1–C-2 bond were co-planar with the ring of mandelate. If, however, the C-2–C-3 bond were to twist such that the transition state lost planarity then the resonance contribution would fall. The inductive effect would, of course, be unaffected by the degree of planarity of the transition state. Thus the fact that the λ value is only 0.64 and not 1.0 is supportive of the idea that the transition state is non-planar.

It is worth considering at this stage that the overall mechanism of catalysis in flavocytochrome b_2, L-LDH, is of the 'Ping-Pong' type [22,23] and this is also likely to operate in *R. graminis* L-MDH. Such a mechanism involves a number of steps which might rate limit the reaction. For example one possibility might be that the reaction is rate limited by reoxidation of the reduced enzyme by the electron acceptor ferricyanide. However, we believe that the data presented in this paper is consistent with the idea that substrate oxidation contributes substantially to overall rate limitation. The fact that $k_{cat.}$ is dependent upon the nature of the substrate (Table 1) and also that log$k_{cat.}$ shows a linear relationship with Hammett–Taft parameters (Figure 5) clearly indicates that substrate oxidation is important in determining the value of $k_{cat.}$ and must therefore contribute to rate limitation.

From all of the above information we envisage the mechanism of mandelate oxidation by L-MDH to proceed as follows: (i) the Michaelis complex is formed; (ii) the enzyme imposes considerable strain on the substrate distorting the geometry at the C-2 position and polarizing the C-2 carbon, this represents the highest energy transition state i.e. the state which rate limits the reaction; (iii) the C-2 hydrogen is lost as a proton generating a carbanion intermediate, this process makes no contribution to rate limitation; (iv) electron transfer from carbanion to flavin then occurs, generating reduced flavin and phenylglyoxalate, the oxidized product. This mechanism has many features in common with that for the oxidation of L-lactate by flavocytochrome b_2, L-LDH, [3,6] but differs significantly at the highest energy-transition state.

Conclusions

From our studies on L-MDH we draw the following conclusions: (i) the oxidations of the substituted mandelates by L-MDH investigated in this present study all proceed via a common mechanism; (ii) the effect of the various ring substituents on the enzyme-catalysed reaction indicates that in the transition state the C-2 carbon is fairly electron rich; (iii) the low resonance contribution is consistent with a non-planar transition state; and (iv) from the KIE evidence it would appear that the L-MDH-catalysed reaction proceeds via a distinctly different transition state from that in L-LDH.

This work was supported by the Science and Engineering Research Council and the University of Edinburgh through funding for the Edinburgh Centre for Molecular Recognition. We thank Professor C. A. Fewson for providing *R. graminis* strain KGX39, and Dr. R. Hrdina, University of Chemical Technology, Pardubice, Czech Republic, for providing help with the isokinetic relationship analysis and for helpful comments.

REFERENCES

1 Fewson, C. A. (1988) FEMS Microbiol. Rev. **54**, 85–110
2 Fewson, C. A. (1992) in The Evolution of Metabolic Function (Mortlock, R. P., ed.), pp. 115–141, CRC Press, Boca Raton, FL, U.S.A.
3 Chapman, S. K., White, S. A. and Reid, G. A. (1991) Adv. Inorg. Chem. **36**, 257–301
4 Smékal, O., Yasin, M., Fewson, C. A., Reid, G. A. and Chapman, S. K. (1993) Biochem. J. **290**, 103–107
5 Labeyrie, F., Baudras, A. and Lederer, F. (1978) Methods Enzymol. **53**, 238–256
6 Reid, G. A., White, S. A., Black, M. T., Lederer, F., Mathews, F. S. and Chapman, S. K. (1988) Eur. J. Biochem. **178**, 329–333
7 Henderson, P. J. F. (1992) in Enzyme Assay: A Practical Approach (Eisenthal, R. and Danson, M. J., eds.), pp. 283–287, IRL Oxford University Press, Oxford
8 Bevington, P. R. (1969) in Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, NY and London
9 Laidler, K. J. (1988) in Chemical Kinetics, 3rd edn., pp. 113–210, Harper and Row, New York
10 Exner, O. (1973) Prog. Phys. Org. Chem. **10**, 413–482
11 Lowry, J. P. and O'Neill, R. D. (1992) Anal. Chem. **64**, 456–459
12 Exner, O. (1964) Collect. Czech. Chem. Commun. **29**, 1094–1113
13 Exner, O. and Beránek, V. (1973) Collect. Czech. Chem. Commun. **38**, 781–798
14 Pytela, O., Večeřa, M. and Vetešník, P. (1981) Collect. Czech. Chem. Commun. **46**, 898–905
15 Livingstone, D. J. (1991) Quantitative Structure–Activity Relationships in Studies in Organic Chemistry, Biochemistry and Related Fields (Zalewski, R. I., Krygowski, T. M. and Shorter, J., eds.), Elsevier, Amsterdam
16 Exner, O. (1988) in Correlation Analysis of Chemical Data, pp. 61–62, Plenum Press, New York/SNTL, Prague
17 Hansch, C., Leo, A. and Taft, R. W. (1991) Chem. Rev. **91**, 165–195
18 Exner, O. (1988) in Correlation Analysis of Chemical Data, pp. 143–144, Plenum Press, New York/SNTL, Prague
19 Ehrenson, S., Brownlee, R. T. C. and Taft, R. W. (1973) Prog. Phys. Org. Chem. **10**, 1–80

20 Craig, P. N., Hansch, C., McFarland, J. W., Martyn, Y. C., Purcell, W. P. and Zahradnik, R. (1971) J. Med. Chem. **14**, 447
21 Exner, O. (1966) Collect. Czech. Chem. Commun. **31**, 3222–3332
22 Tegoni, M., Janot, J.-M. and Labeyrie, F. (1990) Eur. J. Biochem. **190**, 329–342
23 Miles, C. S., Rouvière-Fourmy, N., Lederer, F., Mathews, F. S., Reid, G. A., Black, M. T. and Chapman, S. K. (1992) Biochem. J. **285**, 187–192

Received 4 June 1993/9 August 1993; accepted 8 September 1993