Interaction of Difluoro-oxaloacetate with Aspartate Transaminase

By PATRICIA A. BRILEY, ROBERT EISENTHAL, ROGER HARRISON and
GEOFFREY D. SMITH

Biochemistry Group, School of Biological Sciences, University of Bath, Claverton Down, Bath BA2 7AY, U.K.

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Difluoro-oxaloacetate behaves as a competitive inhibitor of 2-oxoglutarate and as an uncompetitive inhibitor with respect to aspartate in steady-state kinetic experiments with cytoplasmic aspartate transaminase. In the presence of high concentrations of aspartate transaminase, difluoro-oxaloacetate is slowly transaminated to difluoro-aspartate, suggesting its use as a kinetic probe to study the reactions of the aminic form of the enzyme.

Difluoro-oxaloacetate is an isosteric analogue of oxaloacetate in which the CH_2 grouping of oxaloacetate is replaced by CF_2 . Such analogues, containing one or more fluorine atoms in place of corresponding hydrogen atoms of a natural substrate, have been shown in many cases to mimic the biological activity of the latter (Ciba Foundation Symposium, 1972). However, little is known about the interaction of difluoro-oxaloacetate with aspartate transaminase, an enzyme that normally catalyses the transamination of oxaloacetate. The kinetics of aspartate transaminase have been studied in detail by using analogues of the amino acid substrates which give information about the reactions of the aldimine form of the enzyme (Braunstein, 1973). Difluoro-oxaloacetate, which is easily prepared and uncomplicated by enolization, appeared to be a suitable oxo acid analogue with which to examine the lesser-known reactions of the aminic form of the enzyme. The steady-state kinetics of the interaction of difluoro-oxaloacetate with cytoplasmic aspartate transaminase of pig heart are reported in the present paper.

Experimental

Materials

Difluoro-oxaloacetic acid. Difluoro-oxaloacetic acid was prepared by a modification of the method described by Kun *et al.* (1963) in which the geminal hydrogen atoms on the β -carbon of diethyl oxaloacetate are replaced by fluorine by using perchloryl fluoride. NaH was used as a basic catalyst in place of the sodium ethoxide of the earlier method.

A stirred suspension of diethyl oxaloacetate sodium enolate (21 g; Kodak Ltd., Kirkby, Liverpool, U.K.) in toluene/dioxan (2:1, v/v; 100 ml) was cooled to -10°C in an atmosphere of dry CO_2 -free N_2 . Perchloryl fluoride was bubbled into the reaction mixture for 25 min and the temperature was allowed

to rise to between -5° and 0°C . NaH (50% suspension in mineral oil; 4.8 g) suspended in toluene/dioxan (2:1, v/v; 10 ml) was added dropwise to the reaction mixture without interrupting the flow of perchloryl fluoride, which was continued for a further 30 min. After this time gas chromatographic analysis of samples of the reaction mixture showed the content of diethyl difluoro-oxaloacetate to have reached a maximum value. The flow of perchloryl fluoride was stopped and the reaction mixture was purged with CO_2 -free N_2 . Ether (250 ml) and sufficient distilled water to dissolve inorganic salts were added to the reaction mixture followed by saturated aq. NaCl (100 ml). The ether phase was shaken, separated and washed successively with water (200 ml) and saturated aq. NaCl (100 ml). Each aqueous extract was extracted in turn with ether (3×250 ml) and benzene (2×100 ml). All organic extracts were combined, dried (over MgSO_4) and concentrated under diminished pressure giving a brown oil from which an immiscible layer of mineral oil was removed by using a Pasteur pipette. Distillation gave diethyl difluoro-oxaloacetate (9.2 g, yield 41%), b.p. $65\text{--}66^\circ\text{C}/67$ Pa (0.5 mmHg) as a colourless oil, which was homogeneous by g.l.c. Kun *et al.* (1963) quote b.p. $64^\circ\text{C}/134$ Pa (1 mmHg).

The diethyl ester was hydrolysed by boiling a solution of it (9.2 g) in 3M-HCl (60 ml) under reflux for 1 h. The hydrolysate was cooled and concentrated under reduced pressure to give a crystalline residue that was recrystallized from trifluoroacetic acid/benzene to give difluoro-oxaloacetic acid as its monohydrate (5.2 g, yield 68%), m.p. $116\text{--}117^\circ\text{C}$. Titration of the acid with 1M-NaOH gave mol.wt. 186 consistent with its being a monohydrate. Kun *et al.* (1963) give m.p. $119\text{--}120^\circ\text{C}$.

Aspartate transaminase. Cytoplasmic aspartate transaminase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) was prepared in its aldimine form from fresh pig heart ventricles by the method of

Jenkins *et al.* (1959) as modified by Turano *et al.* (1964). 2-Oxoglutarate was included in all buffers. The final product was extensively dialysed against double-distilled water, freeze-dried and stored under vacuum at 5°C. In a typical preparation 2 kg of pig heart ventricles yielded 250 mg of aspartate transaminase (specific activity 640 units/mg) having A_{280}/A_{362} 7.25 at pH 7.4.

The aminic form of the enzyme was prepared from the aldimine form by using cysteine sulphinate as described by Jenkins & D'Ari (1966).

Unless otherwise stated all laboratory reagents were purchased from BDH Chemicals, Poole, Dorset, U.K.

Methods

Enzyme assays. Aspartate transaminase activity was assayed by spectrophotometric determination of oxaloacetic acid at 260 nm at 30°C in 100 mM-KH₂PO₄ adjusted to pH 7.4 with KOH under conditions such that enolization of the oxo form of the acid is not partially rate-limiting (Banks *et al.*, 1963). Standard assay conditions were as described by Banks *et al.* (1968). A cuvette (1 cm path-length) contained L-aspartate (64.5 mM) and 2-oxoglutarate (6.85 mM) in 100 mM-triethanolamine/HCl buffer (final pH 7.5) in a total volume of 3 ml at 25°C. Reactions were initiated by addition of enzyme and were followed by changes in A_{260} . One unit of activity is defined as that amount of enzyme catalysing a change in A_{260} of 0.1/min. Enzyme concentrations are expressed in terms of sites, calculated from protein concentration, determined as described by Banks *et al.* (1968), and a mol.wt. of 46 500 (Ovchinnikov *et al.*, 1973).

Gas-liquid chromatography. G.l.c. was done on columns (2 m × 6 mm) of coiled stainless-steel packed with 3% silicone-gum rubber SE-301 on High Performance (H.P.) Chromasorb W (80–100 mesh). Samples were chromatographed isothermally at 100°C in a Perkin-Elmer F11 gas chromatograph equipped with a flame ionization detector.

N.m.r. spectra were recorded on a Jeol PS 100 n.m.r. spectrometer and the signal-to-noise ratio was enhanced by means of repeated scans with a Jeol JNB SB-3 signal-to-noise booster. Spectrophotometric measurements were performed in a Pye-Unicam SP. 1800 spectrophotometer and free F⁻ was assayed by using a combination fluoride electrode, model 96-09 (Orion Research Inc., Cambridge, MA, U.S.A.). All measurements were done at 30°C unless otherwise stated.

Results and Discussion

In its catalytic role the aminic form of aspartate transaminase interacts with oxo acid substrates and

Scheme 1. Possible reaction scheme for the interaction of difluoro-oxaloacetate with aspartate transaminase

E', Aminic transaminase; E, aldimine transaminase; A, oxo acid substrate (2-oxoglutarate); B, amino acid substrate (aspartate); I, inhibitor (difluoro-oxaloacetate); P, amino acid product (glutamate); Q, oxo acid product (oxaloacetate).

is converted into the aldimine form which reacts with amino acid substrates. The substrate kinetics of the enzyme are known (Braunstein, 1973) to be consistent with a substituted enzyme (Ping Pong) mechanism involving binary complexes. It is also well established (Haarhoff, 1969) that most dicarboxylic acid substrate analogues of aspartate transaminase bind to both forms of the enzyme, as shown for difluoro-oxaloacetate in Scheme 1. The rate equation corresponding to Scheme 1 is:

$$v = \frac{V[A][B]}{[A][B] + K_a[A](1 + [I]/K_i) + K_b[B](1 + [I]/K'_i)} \quad (1)$$

where A, B and I are as defined in Scheme 1, K'_i is the dissociation constant of the aminic transaminase-inhibitor complex, K_i is that of the aldimine enzyme-inhibitor ('abortive') complex, and K_a and K_b are the Michaelis constants for 2-oxoglutarate and aspartate respectively. Eqn. (1) is of the form:

$$v = \frac{V[A][B]}{[A][B] + K'_a[B] + K'_b[A]} \quad (2)$$

where $K'_a = K_a(1 + [I]/K'_i)$ and $K'_b = K_b(1 + [I]/K_i)$. The effect of difluoro-oxaloacetate (I) on K'_a and K'_b was investigated by kinetic studies in which concentrations of 2-oxoglutarate (A) and aspartate (B) were systematically varied at each of a series of concentrations of I. Eqn. (2) may be written as:

$$v = \frac{V[B]}{[B] + K'_b} \frac{[A]}{[A] + K'_a} \quad (3)$$

where

$$K_a^{app} = \frac{K'_a[B]}{[B] + K'_b} \quad (4)$$

Values of K_a^{app} were determined from direct linear plots (Eisenthal & Cornish-Bowden, 1974) of initial rates against concentration of 2-oxoglutarate (A) at different fixed concentrations of aspartate (B) for each difluoro-oxaloacetate concentration. Similar plots of K_a^{app} against aspartate concentration allowed

Fig. 1. Secondary plot of effect of aspartate concentration $[B]$ on K_a^{app} at various inhibitor concentrations

Primary kinetic data were obtained from initial-rate measurements at 2-oxoglutarate concentrations of 0.1, 0.25, 0.5 and 1.0 mM; difluoro-oxaloacetate concentrations: ●, 0; ▼, 25 μM ; ■, 50 μM ; ○, 100 μM . Lines are drawn by using parameters calculated as described in the text. Inset: variation of K_i' as a function of difluoro-oxaloacetate concentration $[I]$.

Fig. 2. Effect of difluoro-oxaloacetate on fractional inhibition of transaminase

Double-reciprocal plot of fractional inhibition (i) against difluoro-oxaloacetate concentration ($[I]$). Aspartate was held constant at 2.0 mM; 2-oxoglutarate concentration: ●, 0.083 mM; ▲, 0.166 mM; ■, 0.33 mM. The enzyme concentration was 1.0 nM. The lines were drawn by using parameters estimated from direct linear plots (Eisenthal & Cornish-Bowden, 1974) of i against $[I]$. Inset: variation of the slope as a function of 2-oxoglutarate concentration ($[A]$).

determination of K_a' and K_b' (eqn. 4). These are displayed in Fig. 1 as plots of $1/K_a^{\text{app}}$ against $1/[B]$ for each value of $[I]$. In such a plot the ordinate intercept is $1/K_a'$ and the abscissa intercept $-1/K_b'$. The lines in Fig. 1 share a common abscissa intercept, but the

ordinate intercepts show a linear dependence on $[I]$ (Fig. 1, inset), showing that K_a' but not K_b' depends on the concentration of inhibitor. It thus appears that the EI complex makes a negligible contribution to the observed rates under the experimental conditions used and suggests that terms in eqn. (1) containing K_i be neglected. This leads to eqn. (5):

$$v = \frac{V[A][B]}{[A][B] + K_a[B](1 + [I]/K_i') + K_b[A]} \quad (5)$$

which adequately describes the results. From this it can be seen that inhibition by difluoro-oxaloacetate is competitive with respect to the oxo acid substrate and uncompetitive with respect to the amino acid substrate. It follows that the species responsible for inhibition is the complex of the inhibitor with the aminic form of aspartate transaminase ($E'I$ in Scheme 1).

Justification for eqn. (5) was provided by a separate series of experiments on the effect of various inhibitor concentrations on the fractional inhibition, i , at different fixed concentrations of 2-oxoglutarate and constant aspartate. The results are shown in Fig. 2 as a plot of $1/i$ against $1/[I]$ where $i = 1 - v_i/v_0$, v_i being the velocity in the presence of inhibitor and v_0 the uninhibited velocity. The observed pattern of straight lines converging on a common ordinate intercept of unity is consistent with both competitive and mixed inhibition. These can be distinguished by a secondary plot of slope against 2-oxoglutarate concentration. Rearrangement of eqn. (1) in fractional inhibition form gives:

$$\frac{1}{i} = \frac{[A][B] + K_a[B] + K_b[A]}{(K_a[B]/K_i') + (K_b[A]/K_i')} \left(\frac{1}{[I]} \right) + 1 \quad (6)$$

If eqn. (6) is applicable, the secondary plot of slope versus $[A]$ will not be linear. However, if $K_i \gg K_i'$ values of K_a and K_b are, in this case, such that the second term in the denominator may be neglected,

$$\frac{1}{i} = \left[\frac{[A]K_i'}{K_a} \left(1 + \frac{K_b}{[B]} \right) + K_i' \right] \left(\frac{1}{[I]} \right) + 1 \quad (7)$$

and a secondary plot of slope against $[A]$ is linear, as observed (Fig. 2, inset). Eqn. (7) may also be directly derived from eqn. (5).

An estimate of $K_i' = 9.2 \mu\text{M}$ was obtained from the plot of K_i' against $[I]$ (Fig. 1, inset). These results are in reasonable agreement with the K_i' of $5.85 \mu\text{M}$ (ordinate intercept) obtained from the secondary plot of slope (Fig. 2) against 2-oxoglutarate (Fig. 2, inset). The kinetic data here, obtained by using purified cytoplasmic aspartate transaminase from pig heart, are in contrast with those obtained by using a crude mitochondrial extract of rat liver, containing aspartate transaminase (Kun *et al.*, 1963), in which difluoro-oxaloacetate was found to behave as a non-

competitive inhibitor with respect to aspartate. Preincubation of transaminase (either form) with inhibitor alone or together with aspartate or 2-oxoglutarate did not affect the initial rates, showing that difluoro-oxaloacetate does not irreversibly inactivate the enzyme.

A significant contribution to the steady-state kinetics from productive breakdown of the difluoro-oxaloacetate-aminic enzyme complex would result in non-hyperbolic variation of initial rates with variation of inhibitor or 2-oxoglutarate concentrations. In neither case was such kinetic behaviour detected, indicating that the measured K'_i is a good approximation to the true dissociation constant of the difluoro-oxaloacetate-aminic enzyme complex. When difluoro-oxaloacetate (0.2mM) was incubated with aspartate (30mM) in the presence of a relatively high concentration (30 μ M) of enzyme, however, a time-dependent increase in A_{260} was observed, indicative of oxaloacetate formation. Detectable rates were obtained only at enzyme concentrations 4000-fold greater than those used in the kinetic studies described above. The turnover was some five orders of magnitude less than those with 2-oxoglutarate, results which are consistent with the assumption that transamination of the fluoroanalogue does not significantly contribute to the steady state kinetics.

Direct spectrophotometric evidence of initial aminic enzyme-difluoro-oxaloacetate complex-formation followed by a very much slower transamination reaction was obtained by incubation of difluoro-oxaloacetate (100 μ M) with aminic enzyme (25 μ M) in 20mM-pyrophosphate buffer, pH7.4. An initial rapid shift ($t_{\frac{1}{2}}$ approx. 5s) of the absorption maximum

of the aminic enzyme (332nm) to lower wavelength (approx. 328 nm) was followed by the slow appearance of an absorption band with $\lambda_{\max}$. 360nm, characteristic of aldimine enzyme, over a period of approx. 30min.

Demonstration of the transamination of difluoro-oxaloacetate to difluoroaspartate was obtained by using cysteine sulphinate as the amino acid substrate. Difluoro-oxaloacetate (50mM), cysteine sulphinic acid (40mM) and aspartate transaminase (0.2mM) were incubated in 50mM-ammonium acetate buffer, pH7.4, for 4 days at 22°C in an n.m.r. tube. The intensity of the ^{19}F n.m.r. single t (Fig. 3) of the oxo acid decreased and a multiplet characteristic of the ABX system of difluoroaspartate (Fig. 3) appeared (J_{AX} 8, J_{BX} 21, J_{AB} 270, $\nu_{\text{A}} - \nu_{\text{B}}$ 456 Hz). Analysis of the completed reaction mixture for free F^- by using an F^- -specific electrode indicated less than 5% decomposition of transaminated difluoro-oxaloacetate. This contrasts with the extensive decomposition reported (Kun *et al.*, 1960) after similar enzymic transamination of monofluoro-oxaloacetate.

Although the reactions of the aldimine form of aspartate transaminase have been extensively studied there is little corresponding knowledge about the aminic enzyme; a circumstance directly attributable to the lack of a suitable substrate analogue (Fasella & Turano, 1970). Apart from a single mention, without experimental details (Kun *et al.*, 1963), there has been no previous report of the substrate activity of difluoro-oxaloacetate with any form of aspartate transaminase, and the present finding that the fluoro analogue is slowly transaminated by the aminic enzyme suggests that difluoro-oxaloacetate may well provide the required probe for kinetic studies of the aminic enzyme.

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Fig. 3. ^{19}F n.m.r. spectra of difluoroaspartate and difluoro-oxaloacetate

The spectra were obtained under the conditions described in the text and are the average of 16 spectral scans. The singlet (----) is the spectrum of unchanged difluoro-oxaloacetate.

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