Reaction of the Aminic Form of Aspartate Transaminase with Difluoro-oxaloacetate

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.

(Received 10 February 1977)

Addition of difluoro-oxaloacetate to the aminic form of aspartate transaminase causes a rapid shift of absorbance maximum of the enzyme from 332 nm to 328 nm, followed by a much slower shift to 360 nm corresponding to complete conversion of the aminic form of the enzyme into the aldimine form or a species with similar spectral parameters in rapid equilibrium with it. Kinetic analysis of both the initial fast reaction and the overall slow reaction by using repeated spectral scanning and stopped-flow techniques allows formulation of a basic reaction mechanism involving at least two intermediate enzyme complexes. Computer simulation of the progress curves of the initial fast reaction based on the suggested reaction mechanism gives kinetic parameters that are consistent with all the data obtained by other methods. A molecular reaction scheme involving a ketimine Schiff-base intermediate is proposed.

Difluoro-oxaloacetate, an isosteric substrate analogue of oxaloacetate in which the CH_2 grouping of oxaloacetate is replaced by CF_2 , has been shown to be a competitive inhibitor of the oxo acid substrate in the transamination reaction catalysed by aspartate transaminase (Briley *et al.*, 1977a). Difluoro-oxaloacetate itself was shown to undergo enzyme-catalysed transamination with aspartate, but this reaction occurred on a time scale that was too slow to affect the inhibition kinetics. Further evidence for transamination was provided by the demonstration of a ^{19}F n.m.r. (nuclear-magnetic-resonance) spectrum corresponding to that expected from difluoro-aspartate after incubation of difluoro-oxaloacetate with the enzyme and an amino acid substrate. Moreover, addition of difluoro-oxaloacetate to the aminic (pyridoxamine) form of aspartate transaminase was reported to cause a slow shift in absorbance maximum from 332 nm to 360 nm characteristic of the aldimine (pyridoxal) form of the enzyme. This slow reaction of difluoro-oxaloacetate suggested the use of the fluoro analogue to help clarify the reactions of the aminic form of aspartate transaminase, which have been less studied than those of the aldimine form.

Materials and Methods

Difluoro-oxaloacetate was prepared as described by Briley *et al.* (1977a); aqueous solutions were adjusted to pH 7.4 with KOH before use. Cysteine-sulphinate was from Sigma (London) Chemical Co., London S.W.6, U.K., and all other reagents were from BDH Chemicals, Poole, Dorset, U.K.

The aminic form of cytoplasmic aspartate trans-

aminase (L-aspartate-2-oxoglutarate aminotransferase, EC 2.6.1.1) was prepared as described by Briley *et al.* (1977a). Concentrations of enzyme are quoted in terms of active sites, assuming a dimer of mol.wt. 93000 (Ovchinnikov *et al.*, 1973). Active sites were determined from A_{332} by using extinction coefficients determined as described by Torchinsky *et al.* (1968).

All spectrophotometric measurements were done on a Unicam SP.1800 instrument unless otherwise stated.

Results

Spectral shift from 332 nm to 360 nm

Addition of difluoro-oxaloacetate to the aminic form of aspartate transaminase resulted in a time-dependent shift of λ_{max} of the absorption spectrum from 332 nm to 360 nm. This change was sufficiently slow to follow the progress of the reaction by repeated spectral scans (Fig. 1). After 75 min the reaction was complete as judged by the disappearance of the absorbance maximum in the 330 nm region and by the fact that addition of 0.5 mM-oxaloacetate (or 2-oxoglutarate) to the completed reaction mixture elicited no further spectral change.

Pseudo-first-order rate constants were calculated from semilogarithmic plots of $A_{360}^0 - A_{360}^t$ against time, obtained from reaction mixtures containing difluoro-oxaloacetate (5–500 μM) and the aminic form of aspartate transaminase (25 μM) in 20 mM-sodium pyrophosphate buffer, pH 7.4 at 25°C. Although the time-dependence of the increase in A_{360} appeared to follow first-order kinetics at all concentrations of

Fig. 1. Spectral changes on addition of difluoro-oxaloacetate to the aminic form of aspartate transaminase

Difluoro-oxaloacetate ($25\text{ }\mu\text{M}$) was added to a solution of the aminic form of aspartate transaminase ($19\text{ }\mu\text{M}$) in 20mM-sodium pyrophosphate buffer, pH7.4, at 25°C . The absorption spectra were obtained from successive scans (scan rate 0.5 nm/s) before (a) and after (b–h) addition of difluoro-oxaloacetate after the time intervals indicated: (b) 0.5 min; (c) 5 min; (d) 9 min; (e) 16 min; (f) 25 min; (g) 45 min; (h) 75 min. —, Baseline.

Scheme 1. Mechanism consistent with the kinetics of the slow spectral change in the reaction of difluoro-oxaloacetate (S) with the aminic form of aspartate transaminase (E')
X, enzyme species absorbing in the 330nm region.

difluoro-oxaloacetate, the apparent first-order rate constant increased with the concentration of difluoro-oxaloacetate becoming independent of the latter at concentrations of difluoro-oxaloacetate greater than $150\text{ }\mu\text{M}$ when the constant reached a limiting value of $1.18 \times 10^{-3}\text{ s}^{-1}$ at 25°C . This behaviour is consistent with a mechanism involving a bimolecular step followed by at least one unimolecular step (Jencks, 1969) as shown in Scheme 1. The occurrence of an intermediate X (Scheme 1) is also indicated by a rapid

initial shift of $\lambda_{\text{max.}}$ from 332 nm to 328 nm on addition of difluoro-oxaloacetate to the aminic form of the enzyme. This rapid shift has been noted previously (Briley *et al.*, 1977a) and is observable in the spectra of Fig. 1. Further evidence for such an intermediate is provided by the absence of an isosbestic point between the species with $\lambda_{\text{max.}}$ 332 nm and that with $\lambda_{\text{max.}}$ 360 nm (Fig. 1), although an isosbestic point does exist between the 328 nm peak and that at 360 nm.

Initial rates of the above spectral change were determined at constant enzyme ($19\text{ }\mu\text{M}$) and varying difluoro-oxaloacetate concentrations ($7\text{--}40\text{ }\mu\text{M}$) in 20mM-sodium pyrophosphate buffer, pH7.4, by following the increase in A_{360} at 25°C . The data were analysed by the mechanism of Scheme 1 assuming a steady state for X. By using the iterative procedure described by Smith *et al.* (1977) values of $K'_m = (k'_{-1} + k'_{+2})/k'_{+1} = 7.0\text{ }\mu\text{M}$ and $k'_{+2} = 1.25 \times 10^{-3}\text{ s}^{-1}$ were obtained.

Changes in absorption maxima of pyridoxal phosphate-dependent enzymes from the 330 nm region to that at 360 nm are known (Fasella & Turano, 1970) to be associated with the conversion of aminic (ketimine) forms of such enzymes into aldimine species. Addition of excess of aspartate or glutamate (100 mM) to a mixture of difluoro-oxaloacetate ($13\text{ }\mu\text{M}$) and the aminic form of the enzyme ($12\text{ }\mu\text{M}$) in 20mM-sodium pyrophosphate buffer, pH7.4, which had been left to react to completion at 25°C , resulted in rapid disappearance of the 360 nm absorption maximum and reappearance of a peak at approx. 330 nm. This indicates the presence of free aldimine enzyme in the reaction mixture at completion.

Spectral shift from 332 nm to 328 nm

It is reasonable to assume that the kinetically predicted intermediate X of Scheme 1 represents the spectrally observable species with $\lambda_{\text{max.}}$ 328 nm, although further intermediates are not precluded. Direct observation of the pre-equilibrium of Scheme 1 is complicated by the ensuing slow shift of the absorbance maximum from 328 nm to 360 nm ($\text{X} \rightarrow \text{Y}$). A close approximation to the pre-equilibrium was obtained in practice, however, in the presence of cysteinesulphinatate, a substrate analogue that reacts rapidly and irreversibly with the aldimine form of aspartate transaminase giving pyruvate and the aminic form of the enzyme (Jenkins & D'Ari, 1966). As shown in Scheme 2, cysteinesulphinatate can be used to recycle the aldimine form of the enzyme to the aminic species. Because the formation of X (Scheme 2) from E' is very much faster than the conversion of X into E virtually all the enzyme will be represented by the pre-equilibrium of Scheme 2, provided that the concentration of cysteinesulphinatate is sufficient to ensure that the recycling of the aldimine species (Scheme 2, step 3) is not rate-limiting. The spectrum

Scheme 2. Reaction sequence used to observe the 328nm spectral peak

S, difluoro-oxaloacetate; A, difluoroaspartate; E' and E, aminic and aldimine forms respectively of aspartate transaminase; K_D , dissociation constant of X (step 1).

obtained under these conditions is shown in Fig. 2 which shows that the 328nm absorption maximum is a well-defined peak with no shoulder at 332nm, indicating that the pre-equilibrium of the aminic form of the enzyme with difluoro-oxaloacetate (Scheme 2, step 1) lies well to the right-hand side. This is consistent with the kinetic parameters derived from initial-rate data for the intermediate X and so further supports the identification of the latter with the species having $\lambda_{\text{max. 328nm}}$. Direct determination of the absorption coefficient at 328nm gave a value of $7.7 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$.

The kinetics of the spectral change from 332nm to 328nm were studied by recording the time course of the change in A_{345} after rapid mixing of the aminic form of aspartate transaminase and difluoro-oxaloacetate in a stopped-flow spectrophotometer. The choice of 345nm was based on the difference spectrum between aminic enzyme and the species with $\lambda_{\text{max. 328nm}}$ (Fig. 2 inset) from which it was found that maximum differences in extinction coefficient occur at 310nm and 345nm. Progress curves were recorded at five different initial concentrations of difluoro-oxaloacetate; the ratio of concentrations of difluoro-oxaloacetate to enzyme varied from approx. 1:1 to approx. 6:1. The progress curve of a typical experiment (Fig. 3) shows a rapid initial decrease in A_{345} followed by a much slower increase (associated with the slow shift from 328nm to 360nm). No transient lag or burst was detected. Observation of the spectral region from 300nm to 500nm during the first 3s of the reaction (at concentrations similar to those used in the stopped-flow experiments) by using a rapid-scanning spectrophotometer (Hollaway & White, 1975) confirmed the absence of a spectrally distinct intermediate between the aminic enzyme and the species with $\lambda_{\text{max. 328nm}}$ on a time scale down to 50ms (Fig. 4).

The amplitude of the initial absorbance change at 345nm was dependent on the difluoro-oxaloacetate concentration (Fig. 5, inset). The data were analysed by assuming that the amplitude can be taken as a measure of a metastable equilibrium between the species with $\lambda_{\text{max. 332nm}}$ and that with $\lambda_{\text{max. 328nm}}$.

Fig. 2. Absorption spectra of aspartate transaminase in the presence of excess of difluoro-oxaloacetate and cysteinesulphinat

(a) Aspartate transaminase ($21 \mu\text{M}$) and cysteinesulphinat (2mM) in 20mM -sodium pyrophosphate buffer, pH 7.4, at 25°C . (b) As for (a) with the addition of difluoro-oxaloacetate (0.2mM final concn.). Inset: difference spectrum between (b) (sample) and (a) (reference).

Fig. 3. Variation of A_{345} on addition of difluoro-oxaloacetate to the aminic form of aspartate transaminase

The reaction mixture contained the aminic form of aspartate transaminase (final concn. $23.1 \mu\text{M}$) from one syringe and difluoro-oxaloacetate (final concn. $80 \mu\text{M}$) from the other in 20mM -sodium pyrophosphate buffer, pH 7.4, at 25°C in a Durrum-Gibson stopped-flow spectrophotometer (light-path 2cm) coupled to a Tectronix storage oscilloscope. Permanent records of the traces were made on Polaroid film.

Application of the iterative procedure described by Smith *et al.* (1977) gave a final plot (Fig. 5) from which a value ($8.75 \mu\text{M}$) for the dissociation constant K_D of

Fig. 4. Rapid spectrophotometric scans of the reaction mixture after addition of difluoro-oxaloacetate to the aminic form of aspartate transaminase

The aminic form of aspartate transaminase (final concn. $100\mu\text{M}$) and difluoro-oxaloacetate (final concn. 1 mM) in 20 mM -sodium pyrophosphate buffer, $\text{pH } 7.4$, were rapidly mixed (3 ms) and the absorption spectrum was scanned (200 nm/ms) after various time intervals as indicated by using the spectrophotometer described by Hollaway & White (1975). (a) 0.125 s ; (b) 0.625 s ; (c) 1.25 s ; (d) 2.625 s .

Scheme 3. Mechanism used as the basis for computer simulation of the fast spectral change in the reaction of difluoro-oxaloacetate (S) with the aminic form of aspartate transaminase (E')

the species with $\lambda_{\text{max. } 328\text{ nm}}$ and a value for the absorption coefficient at 345 nm ($2.8 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) of the same species were derived. The latter value is in good agreement with that ($3.1 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) obtained directly from the spectrum of a mixture of difluoro-oxaloacetate, the aminic form of the enzyme and cysteinesulphinic acid (Fig. 2).

A double-logarithmic plot of the initial rates [obtained from the progress curves by the method of Cornish-Bowden (1975)] of the decrease in A_{345} against initial difluoro-oxaloacetate concentration was linear, with a slope of unity, indicating first-order kinetics with respect to difluoro-oxaloacetate. The apparent second-order rate constant calculated from the intercept was $1.74 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$. However, analysis of the total progress curves by using inte-

grated empirical rate equations showed that the data could not be analysed in terms of simple first- or second-order kinetics and suggested a mechanism for the formation of the species with $\lambda_{\text{max. } 328\text{ nm}}$ involving a bimolecular reaction followed by a unimolecular step. The progress curves were analysed by digital simulation with the program CHEKMAT of Curtis & Chance (1972). This program adjusts the values of specified parameters (usually rate constants) of a reaction mechanism to give the best fit between simulated and experimentally observed time courses. The mechanism (Scheme 3) chosen for simulation was an expanded version of Scheme 1 in which a further intermediate X_1 , spectrally indistinguishable from aminic enzyme, is included before the species with $\lambda_{\text{max. } 328\text{ nm}}$ (redesignated X_2). Because the progress

Fig. 5. Determination of the dissociation constant (K_D) and absorption coefficient ϵ_{345} of the species with λ_{max} . 328 nm (X)

Addition of difluoro-oxaloacetate (S) to the aminic form of aspartate transaminase (E') resulted in an initial rapid decrease in A_{345} (Fig. 3), the amplitude (ΔA_{345}) of which varied with $[S_t]$ (concn. of S at time t) (see inset). By using the procedure of Smith *et al.* (1977), assumed values of K_D ($[E'] [S]/[X]$) were varied until the best fit to a straight line through the origin was obtained when ΔA_{345} was plotted against $[X]$ at different values of $[S_t]$. The above plot represents the best-fit line and corresponds to a value for K_D 8.75 μM . The gradient of the line is $\epsilon'_{345} - \epsilon_{345}$ where ϵ'_{345} is the absorption coefficient of the aminic form of aspartate. Conditions are as described in the legend to Fig. 3 except that difluoro-oxaloacetate concentrations varied from 20 μM to 80 μM .

curves were recorded in absorbance units, the absorption coefficients of the reactants and intermediates at 345 nm were supplied to the program to enable the concentration flux of the various species to be expressed in terms of absorption in the simulation runs.

The absorption coefficient of E' (and hence of X_1) was obtained from direct measurement of the spectrum of aminic enzyme, and that of X_2 from the variation with difluoro-oxaloacetate concentration of the amplitude of the initial decrease in A_{345} as described above. The absorption coefficient for Y was obtained directly from the spectrum of a completed reaction mixture of the aminic form of aspartate transaminase and a small excess of difluoro-oxaloacetate. The value of the first-order rate constant k'_{+2} (Scheme 1) was known both as the limiting first-order rate constant derived from the progress curves of the slow spectral change (332 nm $\rightarrow$ 360 nm) and from analysis of the initial rates of that spectral shift. This value was accordingly assigned to k_{+3} (Scheme 3) as a fixed

parameter. The irreversibility of the step $X \rightarrow Y$ is justified by the total absence of absorbance in the 330 nm region of mixtures of the aminic form of aspartate transaminase and difluoro-oxaloacetate, which had been left to react to completion.

The computer program simulated the progress of the reaction, varying k_{+1} , k_{-1} , k_{+2} and k_{-2} to give the best fit to the experimental progress curves (Fig. 6). The values of the rate constants so obtained are: k_{+1} , $2.61 \times 10^6 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$; k_{-1} , $1.02 \times 10^5 \text{ s}^{-1}$; k_{+2} , $5.57 \times 10^1 \text{ s}^{-1}$; k_{-2} , $1.05 \times 10^{-2} \text{ s}^{-1}$. The form of output of the program CHEKMAT does not allow the definition of precise confidence limits for the rate constants. It does, however, give an indication of the relative precision of these values; from these it can be stated that values for k_{-2} and k_{-1} are more highly defined than those of k_{+1} and k_{+2} .

Although the fit between experiment and theory in Fig. 6 is reasonable, it can be seen that at the highest substrate concentration the fit becomes progressively worse as the reaction proceeds. A possible explanation for this may be the formation of a difluoro-oxaloacetate-alimine enzyme complex (λ_{max} . 430 nm) which is known to absorb only weakly at 345 nm (Briley *et al.*, 1977b). As the reaction is followed as a decrease in A_{345} , the conversion of enzyme into this complex would increase the apparent rate of reaction. This effect would be more pronounced at high concentrations of difluoro-oxaloacetate.

Discussion

The results show that addition of difluoro-oxaloacetate to the aminic form of aspartate transaminase leads to complete conversion of the enzyme into its alimine form or into a species in rapid equilibrium with it. Simulation of the progress curves of the fast initial spectral change (332 nm $\rightarrow$ 328 nm) based on the mechanism shown in Scheme 3 led to a derivation of rate constants k_{+1} , k_{-1} , k_{+2} , k_{-2} which correlate well with independent determinations of related kinetic parameters as follows.

(1) The Michaelis constant $[(k'_{-1} + k'_{+2})/k'_{+1}]$; Scheme 1] derived from the initial rates of the overall slow spectral change (332 nm $\rightarrow$ 360 nm) gave a value of 7.0 μM . In terms of the rate constants of Scheme 3, the Michaelis constant is $(k_{-1}k_{-2} + k_{+2}k_{+3} + k_{-1}k_{+3})/k_{+1}(k_{+2} + k_{-2} + k_{+3})$ giving a predicted value of 7.4 μM .

(2) The dissociation constant of the species with λ_{max} . 328 nm ($[E'] [S]/[X]$; Scheme 2, step 1) obtained from analysis of the amplitudes of the fast spectral change had a value of 8.75 μM , compared with 7.4 μM predicted from the rate constants of Scheme 3 ($k_{-1}k_{-2}/k_{+1}k_{+2}$).

(3) The apparent second-order rate constant derived from the initial rates of the fast spectral change is $1.74 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ compared with a

Fig. 6. Computer simulation of the progress curves of formation of the species with $\lambda_{\max}$ 328 nm

The experimental curves (▲, ●, ■, ▼) are plotted as changes in absorbance (ΔA_{345}) from the absorbance of the free aminic enzyme and were obtained under the conditions described in the legend to Fig. 3, except that the final concentrations of difluoro-oxaloacetate were: ▲, 80 μM ; ●, 50 μM ; ■, 30 μM ; ▼, 20 μM . The simulated curves (Δ , ○, □, ▽) corresponding to curves ▲, ●, ■, ▼ respectively represent the best fit to the experimental curves which were obtained as the rate constants k_{+1} , k_{-1} , k_{+2} , k_{-2} were varied by using the computer program CHEKMAT of Curtis & Chance (1972) run on an IBM 360 computer at University College, London. Note the change of time scale after 15 s.

predicted value of $1.42 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ ($k_{+1}k_{+2}/k_{-1}$).

The internal consistency of the kinetic parameters justifies the mechanism of Scheme 3 as a basis for the reaction of the aminic form of the enzyme with difluoro-oxaloacetate, although further intermediates are not precluded.

The question arises as to the significance in molecular terms of the species in Scheme 3. By analogy with the generally accepted mechanism of transamination (Fasella & Turano, 1970), interaction of difluoro-oxaloacetate and the aminic form of the enzyme might be expected to lead, via a tetrahedral intermediate (I) to a ketimine Schiff base (II) which would undergo a prototropic shift to the aldimine Schiff base (III) (Scheme 4). On the assumption that Y (Scheme 3) is either the aldimine form of the enzyme or a complex in rapid equilibrium with it, then k_{+3} represents the slow step in the interconversion of aminic and aldimine species. With the natural substrates L-glutamate and L-aspartate (Banks *et al.*, 1968) and with *threo*- β -chloroglutamate (Antonini *et al.*, 1970) the rate-limiting step in transamination is believed to coincide with the loss of a proton from the aldimine Schiff base initiating the prototropic shift to a ketimine complex (corresponding to the interconversion III $\rightarrow$ II in Scheme 4). For difluoro-oxaloacetate the evidence presented here is consistent with a similar assignment of the slow step to abstraction of a proton from the Schiff base (II) leading to the aldimine species (III). However, it is also possible that

the rate-determining step could reflect a conformational change of the ketimine Schiff base (II) or even a dehydration of its tetrahedral precursor (I) (cf. Hammes & Haslam, 1969).

On the basis of the above discussion X_2 , the species absorbing at 328 nm (Scheme 3), could represent the ketimine Schiff base (II) or possibly its precursor (I) (Scheme 4). Jenkins (1963) has briefly noted the occurrence of a derivative with absorbance maximum 325 nm on reaction of difluoro-oxaloacetate with the aminic form of aspartate transaminase, and Hammes & Haslam (1969), using temperature-jump techniques, obtained evidence for a ketimine species absorbing in the 330 nm region in the reaction of *erythro*- β -hydroxyaspartate with the aldimine form of the enzyme. However, no spectrally distinct species corresponding to that with $\lambda_{\max}$ 328 nm has been reported with the natural substrates of aspartate transaminase.

It is probable that X_2 is the aminic enzyme-difluoro-oxaloacetate complex responsible for competitive inhibition of transamination of 2-oxoglutarate by aspartate transaminase in the presence of difluoro-oxaloacetate (Briley *et al.*, 1977a). The K_i value kinetically derived for the inhibitory complex was 9.2 μM or 5.9 μM , depending on the experimental approach. These values are compatible with that of 7.4 μM calculated from the rate constants of Scheme 3 [$K_i = k_{-1}k_{-2}/k_{-1}(k_{+2}+k_{-2})$].

Although no further spectrally distinct intermediates could be detected before formation of X_2 ,

Scheme 4. Possible molecular species involved in the reaction of difluoro-oxaloacetate with the aminic form of aspartate transaminase

kinetic evidence indicated the presence of at least one intermediate with $\lambda_{\max}$ 332 nm, which was accordingly included (X_1) in the mechanism of Scheme 3 and in the computer simulation. It is likely that X_1 represents a non-covalent Michaelis complex; this is consistent with an absorbance maximum at 332 nm, as for the aminic enzyme and with a dissociation constant of 39 mM (k_{-1}/k_{+1}).

The second-order rate constant (k_{+1}) describing the rate of formation of X_1 is smaller than the lower limits obtained for the natural substrates ($>10^8$ litre $\cdot$ mol $^{-1}$ $\cdot$ s $^{-1}$). A possible explanation for this could lie in a tendency for difluoro-oxaloacetate to exist in solution as its hydrate (Kun & Dummel, 1969), which would decrease the effective concentration of the reactive keto form.

Whereas the first-order rate constant for the slowest step in the transformation of difluoro-oxaloacetate (k_{+3}) is three orders of magnitude greater than that seen in non-enzymic imidazole-catalysed model systems involving pyridoxal phosphate (Banks *et al.*, 1969), k_{+3} is some five orders of magnitude less than that for the corresponding step in the enzymic transamination of the natural substrates (Fasella & Turano, 1970). There are clearly a number of possible explanations for this discrepancy, depending on the assignment of the rate-determining step. If this is assumed to be the abstraction of a benzylic proton from the ketimine Schiff base (II) as suggested above, then the reaction might be expected on steric grounds to be considerably slower than with oxaloacetate. According to the hypothesis of Dunathan (1966), conversion of aldimine into ketimine Schiff bases is initiated by labilization of the substrate α C-H bond, which must be oriented perpendicular to the π -electron system conjugated to the coenzyme pyridine ring. Attainment of a corresponding orientation of the benzylic proton labilized in the ketimine-aldimine tautomerization would, according to molecular

models, be severely restricted by steric interactions between the fluorine atoms and the benzylic protons in a difluoro-oxaloacetate moiety aligned relative to the cofactor ring as described by Ivanov & Karpeisky (1969). Such steric hindrance, absent with the natural substrate, would serve to account for the very much slower reaction of the fluoro analogue.

The general paucity of knowledge about the reaction of the aminic form of aspartate transaminase has been attributed to the lack of a suitable substrate analogue (Fasella & Turano, 1970). Difluoro-oxaloacetate, which is a stable isosteric analogue, appears to go some way toward fulfilling this need insofar as the slowness of its reaction with the aminic enzyme has provided evidence for the presence of two aminic enzyme-analogue complexes without resort to particularly sophisticated techniques. The direct demonstration of the species with $\lambda_{\max}$ 328 nm, provisionally attributed to a ketimine Schiff base (II), suggests the possibility of trapping this intermediate by borohydride reduction. Moreover, the apparently complete conversion into aldimine species after the slow step of the reaction further suggests a similar trapping and isolation of the corresponding aldimine complex, again from reactions involving initially difluoro-oxaloacetate.

We thank Dr. E. M. Chance and Dr. C. M. Kemp for help in running the programme CHEKMAT, Dr. M. R. Hollaway and Mr. H. A. White for making available the rapid-scanning stopped-flow spectrophotometer, Dr. C. Wharton for use of the stopped-flow spectrophotometer, and the Enzyme Chemistry and Technology Committee of the Science Research Council for a grant (to R. E. and R. H.) and a studentship (to G. D. S.).

References

- Antonini, E., Brunori, M., Fasella, P., Khomutov, R., Manning, J. M. & Severin, E. S. (1970) *Biochemistry* **9**, 1211-1216

- Banks, B. E. C., Bell, M. P., Lawrence, A. J. & Vernon, C. A. (1968) in *Pyridoxal Catalysis, Enzymes and Model Systems* (Snell, E. E., Braunstein, A. E., Severin, E. S. & Torchinsky, Yu. M., eds.), pp. 191–202, Wiley-Interscience, London
- Briley, P., Eisenthal, R., Harrison, R. & Smith, G. D. (1977a) *Biochem. J.* **161**, 383–387
- Briley, P., Eisenthal, R., Harrison, R. & Smith, G. D. (1977b) *Biochem. J.* **163**, 325–331
- Cornish-Bowden, A. (1975) *Biochem. J.* **149**, 305–312
- Curtis, A. R. & Chance, E. M. (1972) *Proc. FEBS Meet. 7th* **25**, 39–57
- Dunathan, H. C. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **55**, 712–721
- Fasella, P. & Turano, C. (1970) *Vitam. Horm. (N.Y.)* **28**, 157–194
- Hammes, G. G. & Haslam, J. L. (1969) *Biochemistry* **8**, 1591–1598
- Hollaway, M. R. & White, H. A. (1975) *Biochem. J.* **149**, 221–231
- Ivanov, V. I. & Karpeisky, M. Ya. (1969) *Adv. Enzymol.* **32**, 22–53
- Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, pp. 572–574, McGraw-Hill, New York
- Jenkins, W. T. (1963) in *Chemical and Biological Aspects of Pyridoxal Catalysis* (Snell, E. E., Fasella, P. M., Braunstein, A. & Rossi Fanelli, A., eds.), p. 146, Pergamon Press, Oxford
- Jenkins, W. T. & D'Ari, L. (1966) *Biochem. Biophys. Res. Commun.* **22**, 376–382
- Kun, E. & Dummel, R. J. (1969) *Methods Enzymol.* **13**, 629
- Ovchinnikov, Yu. A., Egorov, C. A., Aldanova, V. A., Feigina, M. Yu., Lipkin, V. M., Abdulaev, N. G., Grishin, E. G., Kiselev, A. P., Modyanov, N. N., Braunstein, A. E., Polyansky, O. L. & Nosikov, V. V. (1973) *FEBS Lett.* **29**, 31–34
- Smith, G. D., Eisenthal, R. & Harrison, R. (1977) *Anal. Biochem.* **79**, 643–647
- Torchinsky, Yu. M., Malakhova, E. A., Livanova, N. B. & Pikhelgas, V. Yu. (1968) in *Pyridoxal Catalysis: Enzymes and Model Systems* (Snell, E. E., Braunstein, A. E., Severin, E. S. & Torchinsky, Yu. M., eds.), pp. 269–290, Wiley-Interscience, London