Enantiospecific Conjugate Addition of *N*-Nucleophiles to Substituted Fumaric Acids using Methylaspartase

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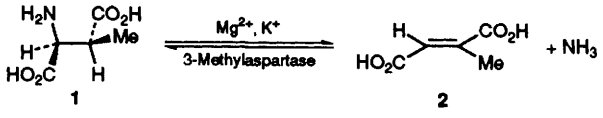
In addition to ammonia, 3-methylaspartase is able to catalyse the conjugate addition of hydrazine, hydroxylamine, methoxylamine and alkylamines to various alkyl- and halogenofumaric acids to give single enantiomers of the corresponding *N*-substituted aspartic acids.

The efficient stereospecific synthesis of small polar biologically active compounds for use as probes in elucidating enzyme mechanism or, receptor site specificity, remains a major synthetic challenge. Commonly problems are associated with the formation of single enantiomers and with the laborious protection and deprotection of key functional groups. Methylaspartase (EC 4.3.1.2) catalyses the deamination of (2*S*,3*S*)-3-methylaspartic acid (Scheme 1).[1]

Previously we had demonstrated that the enzyme was useful in providing access to various 3-substituted aspartic acids *via* the direct amination of substituted fumaric acids.[2] Here we show that a range of *N*-nucleophiles can serve as surrogates for ammonia. We report on the scope and stereospecificity of the conjugate addition reactions and also comment on the geometry of the active site space.

In common with some other ammonia-lyases, the active site of methylaspartase is believed to contain a dehydroalanine residue, derived from Ser-173, to which the amino group of amino acid substrates adds [to form covalent C–N enzyme–substrate adducts (see the reverse of Scheme 2)] prior to the deamination of these substrates.[3–5]

Previous studies in our laboratory had indicated that hydrazine, hydroxylamine and phenylhydrazines could also react with this dehydroalanine residue to cause irreversible inhibition. However, when ^{14}C-*p*-nitrophenylhydrazine† was reacted with the enzyme, the radiolabelled fragment slowly dissociated from the protein during dialysis. This result indicated that the inhibition of the enzyme was not completely irreversible and that for small hydrazines, which would allow the simultaneous cooccupation of the active site by Michael acceptors, it might be possible to synthesise hydrazino acids.



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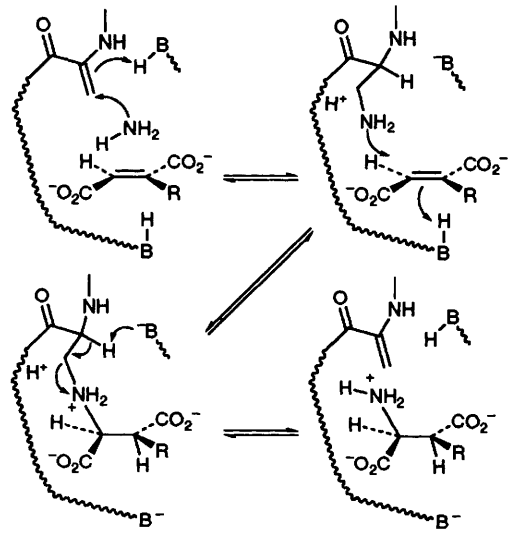
Scheme 1

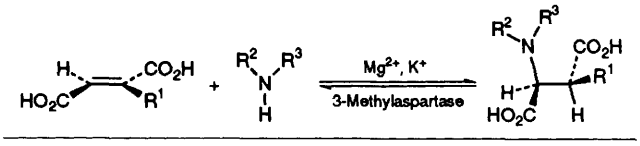


Scheme 2

To test this idea, hydrazine and mesaconic acid were incubated at pH 9.0 with methylaspartase in the presence of potassium and magnesium ions and the reaction was monitored by the periodic removal of aliquots of the solution for analysis by ^{1}H NMR spectroscopy. After several hours new signals appeared at δ 3.55 (1H, d, H$^{\alpha}$), 2.62 (1H, m, H$^{\beta}$) and 0.87 (3H, d, β-CH$_3$). After 3 days, the reaction was complete and ≈90% of the starting material had been converted to the new product (see Table 1). The 2-hydrazino-3-methylsuccinic acid {mp 169–172 °C; $[\alpha]_D$ −18.6 (*c* 0.6, 6 mol dm^{-3} HCl)}, was obtained in 41% yield after recrystallisation from water. Catalytic reduction of a small sample of the compound gave the (2*S*,3*S*) isomer of 3-methylaspartic acid, $[\alpha]_D$ −10.8 (*c* 0.6, water), identical in all respects to an authentic sample.[2] Therefore, the addition of hydrazine to mesaconic acid **2** follows the same stereochemical course as for the addition of ammonia and occurs from the 3-*si*-face in an *anti*-fashion.

In a similar experiment, performed using fumaric acid as the Michael acceptor, 2-hydrazinosuccinic acid was formed in 89% yield and was isolated as the monohydrate in 35% yield {mp 123–124 °C; $[\alpha]_D$ −22.0 (*c* 0.6, 6 mol dm^{-3} HCl)}. Treatment of ethyl- and *n*-propyl-fumaric acid with hydrazine in the presence of methylaspartase also gave the expected hydrazino acid products, Table 1. The 2-hydrazino-3-ethylsuccinic acid was isolated in 50% yield {mp 189–192 °C; $[\alpha]_D$ −13.9 (*c* 0.6, 6 mol dm^{-3} HCl)}.

Table 1 Summary of enzyme catalysed addition reaction[a]



R^1	R^2	R^3	Conversion (%)	Yield (%)
H	Me	H	55	34
Me	Me	H	54	35
Et	Me	H	60	35
Pr^n	Me	H	No reaction	—
Pr^i	Me	H	No reaction	—
H	Me	Me	70	38
Me	Me	Me	No reaction	—
H	NH_2	H	89	35
Me	NH_2	H	91	41
Et	NH_2	H	90	50
Pr^i	NH_2	H	90	—
H	OH	H	90	—
Me	OH	H	90	—
H	Et	H	5	—
H	OMe	H	80	30[b]
Me	OMe	H	70	—
Et	OMe	H	No reaction	—

[a] Some of the reactions were worked-up, as indicated in the text, by acidification of the incubation solution to pH 1, followed by extraction with ether to remove the excess unreacted alkylfumaric acid. Following adjustment of the resulting aqueous solution to pH 3–4, the required addition products crystallised out over several days and were recrystallised from water. All isolated compounds showed the expected spectral and analytical properties. Full characterisations of the other enzymic products will be reported in due course. [b] Isolated as the dimethylester.

Since hydrazine was an effective surrogate for ammonia, the possibility of using hydroxylamine, methoxylamine[6] and alkylamines as the nucleophiles in conjugate addition reactions was tested. Accordingly, fumaric acid was incubated with each of the nucleophiles and in each case reaction occurred to give the corresponding *N*-substituted aspartic acid derivative, see Table 1. (2*S*)-*N*-Methylaspartic acid monohydrate was isolated in 34% yield {mp 189–191 °C; $[\alpha]_D$ +14.4 (*c* 2.0, water at 25 °C)} and was identical in all respects to an authentic sample {mp 189– 190 °C; $[\alpha]_D^{18}$ −15.4 (*c* 0.5, water)[7] for the D-antipode}.

The rate of the additions varied widely for different substrates. However, in general, hydrazine and hydroxylamine were found to be the fastest substrates and dimethylamine the slowest. Curiously, we have found that for incubations involving hydrazine and hydroxylamine, little starting material remains relative to the situation for the addition of simple amines, including ammonia. These observations suggest that the reverse (elimination) reactions occur very slowly and that equilibria favour the addition products.

The enzyme was also able to catalyse the addition of ethylamine to fumaric acid. However, the reaction occurred very slowly, as expected, on account of the steric constraints of the active site. The product was not isolated, due to its low final concentration in the incubation solution, but was characterised by ^{1}H and ^{13}C NMR spectroscopy. The addition of hydrazine and hydroxylamine to chlorofumaric and bromofumaric acid was also catalysed by the enzyme. In the case of the hydrazine addition reactions, the initial products formed, as judged by NMR spectroscopy, were the expected 2-hydrazino-3-halogenosuccinic acids. However, these reacted further before the conjugate addition was complete and could not be isolated.

Remarkably, the enzyme was able to catalyse the addition of dimethylamine to fumaric acid to give the expected product, (2*S*)-*N*,*N*-dimethylaspartic acid. This was isolated in 38% yield {mp 196–198 °C [lit.[8] 198 °C]; $[\alpha]_D$ −2.0 (*c* 0.6, 6 mol dm^{-3} HCl)} and showed the expected spectral and analytical properties. Interestingly, only fumaric acid was able to support this reaction, Fig. 1(*a*). Insufficient space at the active site, and, in particular, unfavourable steric interactions between the *N*-methyl groups and the alkyl group of substituted fumaric acids probably account for the fact that the larger compounds were unable to act as Michael acceptors, Fig. 1(*b*).

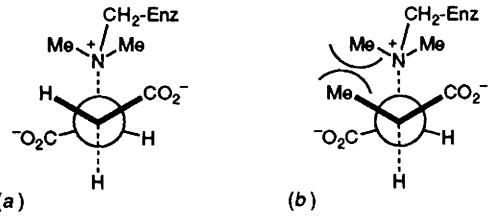


Fig. 1 Expected geometry for the transition states for the enzymic addition of dimethylamine to (*a*) fumaric acid and (*b*) mesaconic acid

Indeed, both *n*-propyl- and isopropyl-fumaric acid, which are known to react with ammonia, failed to react with methylamine although ethylfumaric acid did react. Thus, the active-site space seems to be quite accessible to each of the substrates in a mutually exclusive fashion.

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Footnote

† Prepared from [U–^{14}C]-aniline *via* formylation, nitration, formamide deprotection, diazotisation and finally, reduction of the diazonium salt using sodium sulfite.

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