GLUTAMATE ASPARTATE TRANSAMINASE MODIFIED AT CYSTEINE 390 WITH ENRICHED CARBON-13 CYANIDE[1]

B. Boettcher and M. Martinez-Carrion[2]

Department of Chemistry, Biochemistry and Biophysics Program,

University of Notre Dame, Notre Dame, Indiana 46556

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SUMMARY

The supernatant isozyme of pig heart glutamate aspartate transaminase (EC 2.6.1.1) has been modified "syncatalytically" at cysteine 390 with 90% enriched [^{13}C]cyanide. The C-13 NMR spectra have been recorded under conditions of pH changes and addition of substrates and substrate analogs when the probe environment may be affected. Under these conditions the C-13 probe revealed no changes in the region surrounding cysteine 390.

Supernatant glutamate aspartate transaminase is a dimeric enzyme with identical subunits. Each subunit contains 5 cysteinyl residues which may be classified into at least 3 types according to their reactivity with various sulfhydryl reagents (1). Cysteine 45 and cysteine 82 react with NEM[3] to give an enzyme with 120% the initial specific activity. Cysteine 390 can react "syncatalytically" (2) in the presence of its substrates L-glutamate and α-ketoglutarate with various reagents such as DTNB. The final 2 cysteinyl residues react only under extensive denaturing conditions. Cysteine 390 is particularly interesting because of its probable proximity to the active center and its

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[3]Abbreviations: GAT, glutamate aspartate transaminase; NEM, N-ethylmaleimide; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

unusual property of being "exposed" when its substrates are present (2). Since it is also possible to label cysteine 390 with cyanide while remaining catalytically active (2), 90% enriched [^{13}C]cyanide was used in order to incorporate the C-13 label. It was then possible to investigate the region surrounding cysteine 390 through use of C-13 NMR.

METHODS

GAT, purified as previously described (3,4) was first reacted with NEM and then modified "syncatalytically" with DTNB to form the thionitrobenzoate derivative (2). The thionitrobenzoate group of this mixed disulfide was then displaced with 90% enriched [^{13}C]cyanide giving the thiocyanate derivative.

The number of free sulfhydryls were determined with DTNB in 0.5% sodium dodecyl sulfate, 0.1 M sodium phosphate buffer pH 7.5 using ε_M^{412} = 13600 (5). Radioactivity measurements were determined with a Beckman DPM-100 Scintillation Counter using a 33% Triton X-100/Toluene Cocktail scintillation fluid. Measurements of pH were made with a Radiometer PHM 26 pH meter using a Radiometer GK 2302C combination electrode. Cyanogen bromide cleavage of the modified enzyme and elution on Sephadex G-75 was done according to the procedure of Birchmeier, Wilson, and Christen (2).

C-13 NMR spectra were recorded using the pulsed Fourier transform technique on a Varian XL-100 spectrometer equipped with a Nicolet 1080 digital computer. 12 mm sample tubes were used with a 5 mm coaxial insert tube containing 2% dioxane as an external reference and D_2O for the lock signal. Broad band proton noise decoupling was used for all spectra.

RESULTS AND DISCUSSION

Listed in Table I are the results of modification of glutamate aspartate transaminase at cysteine 390 with [^{13}C]cyanide. Because of the bulkiness and/or charge of the thionitrobenzoate group, this enzyme form has only 5% the initial specific activity. However, along with the release of one thionitrobenzoate group per monomer after the addition of cyanide, the specific activity rose to 60% the initial value. In a parallel experiment [^{14}C] cyanide was also used in the displacement reaction yielding a value of one cyanide moiety incorporated per monomer, which also corresponds to the change in number of free sulfhydryls. The

TABLE I

RESULTS OF MODIFICATION OF GAT AT CYSTEINE 390 WITH [^{13}C]CYANIDE[a]

ENZYME FORM	# FREE SULFHYDRYLS	TNB[b] DISPLACED	^{14}C INCORPORATED	% SPECIFIC ACTIVITY
NEM-GAT[c]	3.0±0.1	------	------	100
TNB-GAT	------	------	------	5
TNB-GAT+^{14}CN	------	1.0	1.1	60
TNB-GAT+^{13}CN	------	0.9±0.1	------	60
TNB-GAT+^{13,14}CN	2.0±0.1	0.9±0.1	1.1±0.1	60

[a]Values in table are given per monomer.

[b]TNB release was followed at 412 nm using ε_M^{142} = 13,600 (5).

[c]The abbreviations used are the following: NEM-GAT: Native GAT modified at its two exposed sulfhydryls with NEM. TNB-GAT: NEM-GAT modified "syncatalytically" at cysteine 390 with DTNB. TNB-GAT + CN: TNB-GAT modified at cysteine 390 by displacing the TNB group with CN.

Sephadex G-75 elution pattern following cyanogen bromide cleavage was similar to that obtained by Birchmeier, Wilson, and Christen (2) showing a single main radioactive peptide fragment.

Figure 1 shows the C-13 NMR spectra of the catalytically active enzyme modified at cysteine 390 with [^{13}C]cyanide. Included on the spectra is the position of a model compound, ethyl thiocyanate, which is located approximately 90 Hz. downfield from the C-13 label. Table II lists the results of these measurements. The concentrations of α-ketoglutarate and L-glutamate are in the "syncatalytic" ratio which was used to expose the residue to modification. The concentration of L-aspartate was chosen to insure the formation of the intermediate binary complexes formed during the transamination reaction (6). α-Methyl-DL-aspartate

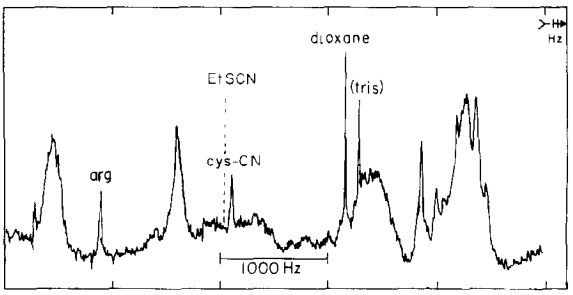

Figure 1. ^{13}C NMR spectra of GAT labelled at cysteine 390 with 90% enriched [^{13}C]cyanide. Shown on the figure are the positions of the guanidino carbon of arginine (arg), the thiocyanate carbon of a model compound ethyl thiocyanate (EtSCN), the 90% enriched [^{13}C]cyanide at cysteine 390 (cys-CN), and the external dioxane reference. The sample concentration was 5 mM (monomer) GAT in 0.2 M KCl, 1 mM EDTA, adjusted to pH 8.3 with Tris base. A 70° pulse (40 usec) was used with 0.4 sec acquisition time. The sweep width was 5000 Hz, and 109,000 transients were accumulated over a 12 hour period.

TABLE II

^{13}C NMR CHEMICAL SHIFT VALUES OF [^{13}C]CYANIDE ON CYSTEINE-390 OF GAT

SAMPLE CONDITION	$[GAT]_{monomer}$ (mM)	Hz FROM ARG[a] (±5)
pH 5.60	2.9	1220
pH 6.70	2.9	1225
pH 7.45	3.2	1225
pH 8.05	3.2	1220
pH 8.90	3.2	1220
2.5 mM α-ketoglutarate 88 mM L-glutamate pH 7.40	5.1	1210
100 mM L-aspartate pH 8.05	4.5	1225
44 mM α-methyl-D,L-aspartate pH 7.45	3.5	1225

[a]No chemical shift was observed with dioxane as external reference.

was added to give a complex which is unable to complete its half reaction, but does form the initial aldimine species (7). The chemical shift of the C-13 label taking the guanidino carbon of arginine, which is unaffected over this pH range (8), as an internal reference, is not significantly affected when the substrates are added "syncatalytically" or when the intermediate enzyme-substrate complexes are formed. In addition, ionization of neighboring residues over the pH range of 5.6 to 8.9 do not affect the chemical shift of the label.

This particular modification has the advantage of being specific for a single residue and allows the incorporation of a highly enriched C-13 label into a catalytically active enzyme. The resulting C-13 NMR spectra gives a single peak which is well isolated from other C-13 peaks in the spectrum. In addition, the particular residue labelled could be expected to undergo significant environmental changes because of its reactivity when its substrates are present. However, under the conditions of these experiments no changes were detected as chemical shifts. The C-13 NMR spectra of other C-13 nuclei show chemical shift sensitivity only when nearby ionizing atoms are present (8,9,10,11). Thus the chemical shift insensitivity of the [^{13}C]cyanide probe, may be an indication of its limited usefulness as a probe of protein structure and function in localized regions of some enzymes. On the other hand, steric hindrance differences in the reactivity of cysteine 390 with sulfhydryl reagents between the free enzyme and enzyme-substrate complexes without localized environmental changes in the region of the C-13 probe may indeed occur. This could also explain the lack of variation in the resonance of the C-13 signal under a variety of experimental conditions.

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