A NUCLEAR MAGNETIC RESONANCE STUDY OF THE BINDING OF SUBSTRATE ANALOGUES TO A MODIFIED ASPARTATE TRANSCARBAMYLASE

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Abstract:

Nuclear magnetic resonance methods have been used to determine the relationship between the modification of a specific residue in the active site of the enzyme aspartate transcarbamylase with a chromophoric mercurial and changes occurring in the binding of substrate analogs to the active site. The binding of succinate, an analog of the substrate L-aspartate, to the modified enzyme is completely blocked where as the binding of acetyl phosphate, an analog of the substrate carbamyl phosphate, is only slightly perturbed.

One approach to the study of enzyme mechanisms is the modification of amino acid side chains with group specific reagents. A change in activity brought about by a selective modification of the protein can occur through the disruption of the interactions involved in binding and/or catalysis. The location of a modified residue could be sufficiently close to the active site to sterically interfere with the binding and catalytic processes. Alternatively the modification could be at a location far removed from the active site but affect activity through a conformational change in the protein.

Using NMR techniques we have determined the relationship between the modification of a specific residue and the changes occurring in the binding of substrate analogs to the active site of the enzyme aspartate transcarbamylase* (Ec 2.1.3.2.). The regulatory enzyme ATCase from E.coli reacts with the chromophoric mercurial 2-chloromercuri-4-nitrophenol (1) with loss of enzymatic activity (2). The studies of Wiley et al (3) have confirmed that the mercurial

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*Abbreviations. ATCase: Aspartate Transcarbamylase, CAP: Carbamyl Phosphate, AP: Acetyl Phosphate, R: Regulatory Chain of ATCase, C: Catalytic Chain of ATCase, β-ME: β-mercaptoethanol.

is bound at a single site, that is six per hexamer $(RC)_6$. The mercurial derivative is isomorphous with the native protein suggesting that there is a minimal perturbation of the structure, at least to 5 Å resolution, on binding of the mercurial. The mercurial binds to the single thiol of the C-chain (2). This thiol is thought not to be directly involved in catalysis as Vanaman & Stark (4) were able to selectively modify this residue in the isolated C-chain without inhibiting activity.

We have used the NMR techniques developed by Schmidt et al (5) and Sykes et al (6) to compare the binding of substrate analogues to the modified and unmodified enzyme, and therefore determine the stereochemical relationship of the sulfhydryl group to the active site.

Experimental:

ATCase was prepared by the method of Gerhart and Holoubek (7). Modified ATCase was prepared by reacting 2-choromercuri-4-nitrophenol in a 1:1 ratio with (RC) units of ATCase (MW $\simeq$ 50,000); the ATCase concentration was typically 15 mg/ml in 0.1 M Triethanolamine buffer adjusted to pH 7.4 with HCl. The activity of the enzyme was checked using a pH stat assay (8) to determine that the enzyme was > 95% inactivated. Activity was immediately restored both at low (1-2 μg/ml) and high (~10 mg/ml) enzyme concentration upon the addition of β-ME(5mM) to the solution.

NMR spectra were obtained with a Varian HA-100 spectrometer at ambient temperature(33°C) using an external capillary of hexamethyldisiloxane as a lock signal. Acetone ($\lesssim$.25% v/v) was used as an internal standard. Time averaged spectra were obtained using a Varian 620i computer (9).

The linewidth of the succinate resonance was obtained by subtracting the linewidth of the acetone internal standard from the observed linewidth. The linewidth of the acetyl phosphate resonance was obtained by fitting the observed lineshape as the sum of two Lorentzian resonances separated by 0.90Hz ($J_{\underline{CH}_3, {}^{31}P}$ (5)) using a non linear least squares program with the linewidth of the indivual resonances as a parameter of the fit (10), and then subtracting

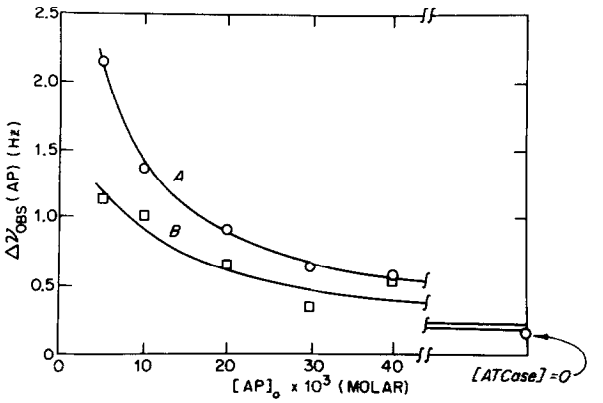

Figure 1: The observed linewidth of the individual resonances of the acetyl phosphate doublet as a function of initial AP concentration (a) in the presence of 10mg/ml ATCase at pH = 7.4. The solid line through the experimental points was calculated with $K_D = 2.5 \times 10^{-3}$M, $\Delta\nu_{EI} = 72$ Hz, $\Delta\nu_I = 0.23$ Hz (b) in the presence of 10mg/ml modified ATCase at pH = 7.4. Same buffer as in Figure 2. The solid line through the experimental points was calculated with $K_D = 9.3 \times 10^{-3}$M, $\Delta\nu_{EI} = 74$ Hz, $\Delta\nu_I = 0.15$ Hz.

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the linewidth of the acetone internal standard from the linewidth obtained.

The pH of the solutions (meter reading for D_2O solutions) was determined using a Radiometer pH meter standardized at pH = 7.0.

RESULTS:

The linebroadening of the individual resonances of the acetyl phosphate doublet (see experimental section) as a function of acetyl phosphate concentration in the presence of native enzyme and in the presence of modified enzyme at pH = 7.4 are shown in figure 1. The observed linebroadening was fitted to the function

$$\Delta\nu_{OBS} = \frac{[EI]}{I^\circ}\Delta\nu_{EI} + \frac{[I]}{I^\circ}\Delta\nu_I \qquad (1)$$

with

$$\frac{[EI]\,[I]}{[EI]} = K_D \qquad (2)$$

using nonlinear least squares techniques (10), with the bound linewidth $\Delta\nu_{EI}$ and the dissociation constant K_D as iterative parameters. The results obtained

TABLE I. Linebraodening of succinate at pH 7.4, 0.1M imidazole-HCl buffer, D_2O 1mM EDTA

	$\Delta\nu$(succ)
a) 15 mM succinate, 25mM AP, and 10mg/ml ATCase.	0.1 ± 0.2
b) 15mM succinate, 25mM AP, 5mM CAP and 10mg/ml ATCase.	2.9 ± 0.2
c) 15mM succinate, 5mM CAP and 10mg/ml modified ATCase.	0.1 ± 0.2
d) 15mM succinate, 5mM CAP and 10mg/ml ATCase.	3.2 ± 0.2

* Sample (b) was obtained from sample (a) by adding CAP to the NMR tube.

** Sample (d) was obtained from sample (c) by the addition of β-ME to the solution.

were $K_D = (2.5 \pm 1.0) \times 10^{-3}$ M and $\Delta\nu_{EI} = 72 \pm 11$ Hz for the native enzyme and $K_D = (9.3 \pm 5.6) \times 10^{-3}$ M and $\Delta\nu_{EI} = 74 \pm 24$ Hz for the modified enzyme. Essentially identical results for the dissociation constants have been obtained using optical techniques (11). Upon the addition of CAP to the AP + enzyme solutions, the linebroadening of the AP resonance was greatly diminished because of the competition of CAP and AP for the same binding site (K_D (CAP)<< K_D(AP) (12)).

The results for succinate are presented in Table I. With native ATCase at pH 7.4 there is no linebroadening of succinate in the absence of CAP. There is also little or no linebroasening in the presence of AP or P_i, but considerable linebroadening in the presence of CAP. This is in agreement with Schmidt et al (5) who showed for the C subunit that the succinate resonance was broadened significantly in the presence of AP above pH 7.0.

Mercurial inhibition of the enzyme results in complete abolition of the succinate linebroadening in the presence of CAP. In all cases the inhibition by mercurial was completely reversible as evidenced by the linebroadening of succinate in the presence of CAP upon the addition of β-ME to the mercurial enzyme.

Similiar results were obtained over the pH range 7.0 to 8.5 and also for the modified and unmodified C subunit.

Discussion:

The linebroadening of the AP resonance in the presence of native ATCase ($\Delta\nu_{OBS}$) is the result of the rapid exchange of AP between solution and the catalytic sites of ATCase (5) (equation 1). The linewidth of bound AP is much greater than that of AP in solution because of the restricted mobility of AP when bound in the catalytic site. The addition of the mercurial derivative to the enzyme has no effect upon the bound linewidth for acetyl phosphate but increases the dissociation constant for AP by about a factor of 3. This implies that the mobility of the CH_3 group of acetyl phosphate when bound to the modified and native enzymes is the same even though the binding is weaker to the modified enzyme. A rotational correlation time of 5×10^{-8} sec. for AP bound to ATCase can be calculated by assuming that the nuclear spin relaxation is dominated by intramolecular dipole-dipole interactions and that the internal rotation of the methyl group of AP is fast.[1] This rotational correlation time is of the magnitude expected for a molecule of the size of ATCase (6).

The linebroadening of succinate in the presence of CAP and the native enzyme is the result of the slow exchange of succinate between solution and the CAP-ATCase-succinate complex (6)

$$\Delta\nu_{OBS} = \Delta\nu_F + P_B \frac{k_{-1}}{\pi} \qquad (3)$$

where k_{-1} is the rate constant for the dissociation of succinate from the ternary complex. The observation of zero linebroadening in the presence of the modified enzyme implies that the succinate site is blocked or that k_{-1} is smaller (i.e. tighter binding) for the modified enzyme. Equilibrium dialysis

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1. The expression for the rotational correlation time of bound AP (τ_{MACRO}) in the limit that the nuclear spin relaxation is dominated by intramolecular dipole-dipole interactions and is not in the extreme narrowing limit ($(\omega_o\tau_{MACRO})^2>>1$), and that the internal rotation of the $-CH_3$ group is fast ($\tau_{int} << \tau_{MACRO}$), is given by (13)

$$\frac{1}{T_2} = \frac{9}{10} \frac{(h^2\gamma^4)}{r^6} \left(\frac{1 - 3\cos^2\theta}{2}\right)^2 \tau_{MACRO}$$

where $\theta=90^o$.

studies have shown that succinate is not bound to the modified enzyme (2), eliminating the hypothesis that the decrease in linebroadening is due to a slower off rate constant for succinate.

Vanaman and Stark (4) have shown that the SH group which is being modified is near to the active site but not involved in catalysis. We have shown that the proximity to the active site is such that the addition of a label the size of the mercurial used in this study blocks the binding of succinate , but only slightly perturbs the degree of AP binding. The fact that similar results are obtained for the catalytic subunit implies that we are looking at the catalytic site with no interference from binding to the regulatory subunit.

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