KINETIC AND BINDING EFFECTS OF 1,N^6-ETHENOADENOSINE TRIPHOSPHATE TO ASPARTATE TRANSCARBAMYLASE

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SUMMARY

A fluorescence polarization study reveals that 1 N^6-ethenoadenosine triphosphate (εATP) binds to native aspartate transcarbamylase (ATCase) from E. coli. However, unlike adenosine triphosphate (ATP) εATP inhibits the enzyme, which strongly suggests that the N-1 atom in the purine ring is crucial for ATP activation. Study of the binding curve for εATP shows multiple binding sites with overlapping affinities, but a simple system of six equivalent binding sites with a dissociation constant $K = 7.5 \times 10^{-5}$M gives a reasonable approximation to the experimental data.

Aspartate transcarbamylase (ATCase) from E. coli catalyzes the formation of carbamyl aspartate from carbamyl phosphate and aspartate in the pyrimidine biosynthetic pathway. It is subject to activation and inhibition by nucleotide effectors which bind to the enzyme at sites distinct from the active sites (1). Previous work (1) (2) show that adenosine, adenosine monophosphate as well as adenosine triphosphate are the only activators among the nucleosides and nucleotides. (1) (3) (4). The binding studies of the nucleotides with the enzyme have been concentrated on cytidine triphosphate (CTP) binding (5) (6) (7). Winlund and Chamberlin (7) reported that there are six binding sites for CTP which fall into two equal classes differing 43-fold in their affinities for CTP.

1, N^6-ethenoadenosine triphosphate, abbreviated as εATP, is a highly fluorescent analogue of ATP (18). It is able to substitute for ATP showing activity in phosphoryl, pyrophosphoryl, and adenylyl transfer enzyme systems. It also acts as allosteric effector in the phosphofructokinase system (18).

In this report we studied the effect of εATP on ATCase kinetics and its binding to the enzyme using fluorescence techniques.

MATERIAL AND METHODS

ATCase was isolated and purified according to the method of Gerhart and

Holoubek (9). The purity of the enzyme preparation was checked by using polyacrylamide gel electrophoresis.

Dilithium carbamyl phosphate was from Sigma Company. L-aspartic acid uniformly labeled with C^{14}, specific activity about 150 mC per m mole was purchased from New England Nuclear. All common chemicals were commercial reagent grade materials, used without further purification, with the exception of imidazole which was recrystallized twice. εATP was dissolved in 0.1 M imidazole-HOAc pH 7 buffer, the buffer used in the enzyme assay, as stock solution and added in small aliquots to the enzyme activity assay reaction mixtures to reach the final desired concentrations.

Enzyme assay was carried out by using the method of Porter et al (10). The reaction mixtures contained ∿0.1 μg/ml of ATCase, 3.6 mM dilitium carbamyl phosphate and varying amount of radioactive aspartate. The concentration of εATP used was 2 mM.

The fluorescence intensity measurements were done with the instrument described by Weber and Young (11). Monochromatic light from a Xenon lamp was split into two beams, of which one was scattered and served as a monitor on fluctuations in light intensity and another used to excite the sample. The fluorescence was excited at 310 nm and the emission at 405 nm was measured. The band widths of excitation and emission were 3.33 nm and 6.66 nm, respectively.

Fluorescence polarization measurements were carried out on the instrument described by Weber and Bablouzian (12). The 310 nm exciting light was isolated by means of a Bausch and Lomb grating monochrometer. The emission was filtered through 2 mm of 0.1 M $NaNO_2$ and Corning glass CS3-75 filters.

All measurements were obtained through a Dana Ratio Digital Voltmeter, and averaged by using an average-and-standard deviation program on a Hewlett-Packard 9100A calculator. All the measurements were carried out at 4°C.

The polarization of εATP in the solution in the presence of ATCase is the average of the free and the bound weighted according to their respective intensities. The fluorescence intensity of the εATP solution is designated I_o, that of

the εATP-ATCase solution I. The polarization of free εATP is P_F, that of εATP when it is completely bound to protein is P_B. The fraction of εATP bound is x. Then the observed polarization P of the εATP-ATCase system is

$$P = \frac{(1-x)I_o P_F + [I - (1-x)I_o]P_B}{I}$$

and x can be solved as

$$x = 1 - \frac{I}{I_o}\left(\frac{P_B - P}{P_B - P_F}\right)$$

P_B was obtained by plotting $\frac{1}{P}$ versus $\frac{1}{[ATCase]}$ and extropolating to $\frac{1}{[ATCase]} \to 0$. Because in the measurements of polarization, the exciting light was polarized in a plane perpendicular to the axis of observation, but in the fluorometer the exciting light was unpolarized, the observed fluorescence intensity in the fluorometer should be corrected as follows: Suppose the fluorescence intensity excited by light polarized perpendicular to the axis of observation is I, then $I = I_1 + I_2$ where I_1 and I_2 are the intensities of the components of fluorescence parallel and perpendicular to the exciting vector. If the intensity upon unpolarized excitation is I', $I' = \frac{1}{2} I_1 + \frac{3}{2} I_2$. By definition the polarization p, $p = \frac{I_1 - I_2}{I_1 + I_2}$ so the observed fluorescence intensity in the fluorometer I' should be corrected as $I = \frac{I'}{1 - \frac{1}{2}p}$.

From x, the number of mole of εATP bound per mole of ATCase ($\bar{n}$) can be calculated as $\bar{n} = x \cdot \frac{X_o}{P_o}$ where X_o and P_o are the total εATP and total ATCase concentrations respectively. The concentration of the free εATP, X, is then calculated from the relation $X = X_o - \bar{n}P_o$. The computation and plotting of the titration curve of Figure 2 was done by an IBM 360/75 computer system.

RESULT AND DISCUSSION

The results from enzyme kinetic activity assay are shown on Figure 1. εATP is not an activator. It inhibits the enzyme. In the presence of εATP, the K_m, or the concentration of substrate for half saturation for aspartate is increased for that molecule. But the Vmax of the system is unchanged. This effect is similar to the effects caused by other nucleotide inhibitors (1) (13). With 2 mM εATP, the shift of the saturation curve is similar to that of 0.5 mM CTP (1) (13).

Recently London and Schmidt (2) proposed that the nucleotide binding site in the

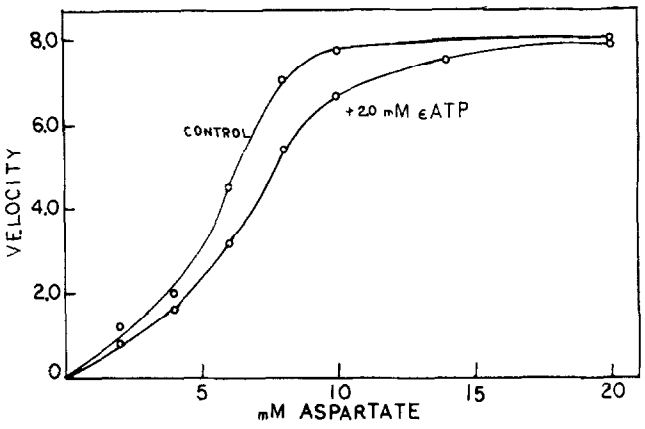

Fig. 1.

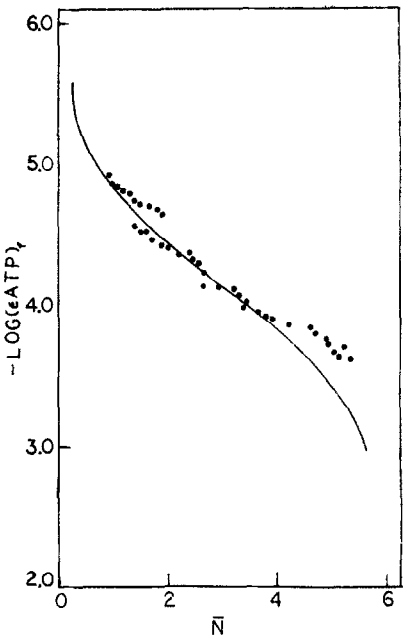

Fig. 2.

Figure 1 - Effect of εATP on the kinetics of ATCase. Reaction mixtures contained 3.6×10^{-3} M carbamyl phosphate; aspartate varied as indicated; 2×10^{-3} M εATP when used; 0.1 M imidazole HOAc, pH 7 buffer and 11.2×10^{-2} µg of enzyme protein per ml. Velocity = units of activity per mg of protein $\times 10^{-3}$.

Figure 2 - Binding of εATP to ATCase. Experiments were carried out at 4°C in a buffer contained 0.04 M potassium phosphate pH 7; 2×10^{-3}M 2-mercaptoethanol and 2×10^{-4} M EDTA. The experimental points are represented by ● ● ● . Theoretical calculation is indicated by ——— .

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protein contains both an electrophilic moiety, capable of binding to a basic ring nitrogen of the nucleotide, and a hydrogen-bond receptor. Since the most basic center of ATP in the ring is N-1, they proposed that the activation by ATP is due to binding in its anti conformation to an expanded conformation of ATCase through an electrophilic interaction with N-1 and a hydrogen bond with the amino group at C6. The most basic center in the inhibitor GTP ring structure is N-7, so that N7, instead of N-1, binds to the electrophilic portion in the contracted conformation of the site. It is clear in the case of εATP that the N-1 atom is no longer basic in nature, and the amino group at C6 is engaged in another ring. The inhibitory action of εATP is then consistent with the London and Schmidt proposal. Besides, the P_B value obtained was 0.19. The

polarization of εATP measured in propylene glycole at -30°C is 0.32. If we take this value as the limiting polarization, then from the equation (14) $(\frac{1}{P_B} - \frac{1}{3}) = (\frac{1}{P_o} - \frac{1}{3}) \times (\frac{2}{3\cos^2\omega - 1})$, the average angle of free rotation is determined as $\omega \simeq 33°$. This large rotation could not possibly be contributed by the rotation of the whole enzyme molecule during the 20 n sec lifetime of the chromophore. A fluorophore of 20 n sec lifetime attached to a spherical protein of molar volume 3×10^5 ml would on average rotate 12° during the excited state. It is reasonable to conclude that the εATP can undergo independent rotation and the base part of it would not associate with the protein through multiple points of attachment.

The result of εATP binding with ATCase in 0.04 M KPO_4 pH 7 buffer 2×10^{-4} M NaEDTA, 2×10^{-3} M 2 mercaptoethanol is presented in Figure 2. It clearly shows that there are multiple binding sites with overlapping affinities. The solid line in Figure 2 is a theoretically calculated binding curve for the system of six equivalent binding sites with a dissociation constant $K = 7.5 \times 10^{-5}$ M. The experimental points fit well the theoretical curve except at high ligand concentrations. According to Porter et al (10), any phosphate containing compound can bind to the carbamyl phosphate site on the catalytic subunits with dissociation constants in the range of 10^{-3} - 10^{-4} M. It is reasonable to assume that εATP will bind at both the regulatory and catalyic sites at high concentration. The deviation of the experimental points from the theoretical curve at high εATP concentration could be due to such non-specific binding at the catalytic subunits, but we cannot rule out the possibility of two classes of binding sites at the regulatory subunits. It is hard to determine whether there are two classes of binding sites, for the dissociation constant for the low affinity site would be similar to that of the non-specific binding at the catalytic subunits. However, a unique dissociation constant of 7.5×10^{-5} M for the six equivalent binding sites is a good approximation to the binding strength of the system.

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