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Lysine-60 in the Regulatory Chain of *Escherichia coli* Aspartate Transcarbamoylase Is Important for the Discrimination between CTP and ATP†

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ABSTRACT: Lysine-60 in the regulatory chain of aspartate transcarbamoylase has been changed to an alanine by site-specific mutagenesis. The resulting enzyme exhibits activity and homotropic cooperativity identical with those of the wild-type enzyme. The substrate concentration at half the maximal observed specific activity decreases from 13.3 mM for the wild-type enzyme to 9.6 mM for the mutant enzyme. ATP activates the mutant enzyme to the same extent that it does the wild-type enzyme, but the concentration of ATP required to reach half of the maximal activation is reduced approximately 5-fold for the mutant enzyme. CTP at a concentration of 10 mM does not inhibit the mutant enzyme, while under the same conditions CTP at concentrations less than 1 mM will inhibit the wild-type enzyme to the maximal extent. Higher concentrations of CTP result in some inhibition of the mutant enzyme that may be due either to heterotropic effects at the regulatory site or to competitive binding at the active site. UTP alone or in the presence of CTP has no effect on the mutant enzyme. Kinetic competition experiments indicate that CTP is still able to displace ATP from the regulatory sites of the mutant enzyme. Binding measurements by equilibrium dialysis were used to estimate a lower limit on the dissociation constant for CTP binding to the mutant enzyme ($>1 \times 10^{-3}$ M). Equilibrium competition binding experiments between ATP and CTP verified that CTP still can bind to the regulatory site of the enzyme. For the mutant enzyme, CTP affinity is reduced approximately 100-fold, while ATP affinity is increased by 5-fold. These data imply that lysine-60 in the regulatory chain of aspartate transcarbamoylase is partially or perhaps fully responsible for the enhanced binding of CTP over ATP to the wild-type enzyme and is partially responsible for the discrimination between these nucleotides.

Aspartate transcarbamoylase (EC 2.1.3.2) catalyzes the first reaction of the pyrimidine biosynthesis pathway, the carbamoylation of the amino group of aspartate by carbamoyl phosphate. In *Escherichia coli*, this enzyme is subject to activation by ATP and feedback inhibition by CTP and UTP, the end products of the pathway (Gerhart & Pardee, 1962, 1963; Wild et al., 1989; Yates & Pardee, 1956). The holoenzyme,[1] composed of two trimeric catalytic subunits and three dimeric regulatory subunits, exhibits homotropic cooperative interactions for aspartate binding. The catalytic subunit, which carries the active sites and is insensitive to ATP, CTP, and UTP, shows Michaelis–Menten kinetics. The regulatory subunit exhibits no catalytic activity but binds the allosteric effectors. At pH 7.0, the combination of CTP and UTP will

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[1] Abbreviations: T and R states, tight and relaxed states of the enzyme having low and high affinity, respectively, for the substance; C–R, interface between the catalytic and regulatory subunits of aspartate transcarbamoylase; $[S]_{0.5}^{Asp}$, aspartate concentration at half the maximal observed specific activity; holoenzyme, entire aspartate transcarbamoylase molecule composed of two catalytic subunits and three regulatory subunits.

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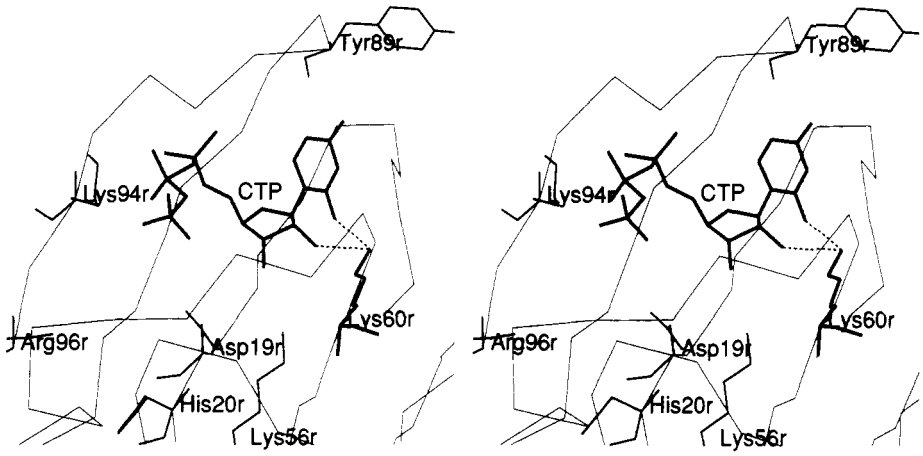

FIGURE 1: Stereoview representing selected side chains in the binding site of CTP. The CTP nucleotide (dark) bound to the regulatory site in chain R1 as well as nearby amino acid residues is shown. Asp-19r, Lys-60r, and Tyr-89r interact with the pyrimidine ribose moiety, whereas His-20r, Lys-56r, Lys-94r, and Arg-96r are closer to the triphosphate moiety. Lys-60r (dark) interacts specifically with the 2-keto group of the pyrimidine ring and the 2′-OH group of the ribose of CTP. The data used to draw this figure are from Kim et al. (1987).

inhibit the enzyme to a greater extent than CTP alone; however, UTP alone does not inhibit the enzyme (Wild et al., 1989).

Detailed structural information is now available concerning the unliganded holoenzyme as well as the holoenzyme complexed with CTP and a variety of substrate analogues (Gouaux & Lipscomb, 1988; Honzatko et al., 1982; Honzatko & Lipscomb, 1982; Ke et al., 1984; Kim et al., 1987; Krause et al., 1985, 1987; Voltz et al., 1986). Together, kinetic (Gerhart & Pardee, 1963, 1964; Ladjimi et al., 1985; Thiry & Hervé, 1978), binding (Changeux et al., 1968), and crystallographic studies (Honzatko & Lipscomb, 1982) indicate that ATP and CTP bind competitively to the same site but they produce opposite effects on the homotropic cooperativity and on the apparent K_m for the substrates (Gerhart & Pardee, 1964).

CTP binding to the regulatory sites is complex; two classes of regulatory binding sites having differing affinities for CTP can be distinguished (Allewell et al., 1975; Burz & Allewell, 1982; Gray et al., 1973; Matsumoto & Hammes, 1973; Tondre & Hammes, 1974; Winlund & Chamberlin, 1970). This observation is often interpreted in terms of ligand-induced negative cooperativity (Honzatko et al., 1982; Kim et al., 1987; London & Schmidt, 1974; Tondre & Hammes, 1974), although other data were interpreted on the basis of an intrinsic asymmetry in the enzyme (Suter & Rosenbusch, 1976, 1977). Binding of ATP follows a pattern similar to that of CTP with two classes of affinity sites, except that binding to the regulatory site is about an order of magnitude weaker than that of CTP (Matsumoto & Hammes, 1973). Both ATP and CTP, like any phosphate-containing compound, compete directly with carbamoyl phosphate for binding at the active (Porter et al., 1969), and crystallographic evidence exists for their actual binding to this site (Honzatko & Lipscomb, 1982; Kim et al., 1987).

Analysis of the X-ray structure of the holoenzyme with CTP bound (Kim et al., 1987) indicates that Lys-60r[2] interacts with the 2-keto group on the pyrimidine ring as well as the 2′-OH of the ribose of CTP (see Figure 1). Since both CTP and UTP have a keto group at the 2 position and ATP has no analogous functionality, it was of interest to determine the importance of the interaction between Lys-60r and the pyrimidine nucleotides. Therefore, using site-specific mutagenesis, we replaced Lys-60r with Ala (Lys-60r → Ala),[3] and we present here the analysis of this mutant version of aspartate transcarbamoylase.

Experimental Procedures

Materials

ATP, CTP, carbamoyl phosphate, *N*-carbamoyl-L-aspartate, L-aspartate, potassium dihydrogen phosphate, imidazole, and Tris were purchased from Sigma. [^{3}H]ATP and [^{3}H]CTP were purchased from NEN Research Products. The carbamoyl phosphate was purified by precipitation from 50% (v/v) ethanol and stored desiccated at −20 °C (Gerhart & Pardee, 1962). Enzyme-grade ammonium sulfate was purchased from ICN Biochemicals. The plasmid pUC119 and the phage M13K07 were a gift of J. Messing, Rutgers University.

Methods

Oligonucleotide Synthesis. The oligonucletoide required for the site-specific mutagenesis as well as the sequencing primers was synthesized by using an Applied Biosystems 381A DNA synthesizer.

Wild-Type and Mutant Enzyme Purification. The wild-type and Lys-60r → Ala aspartate transcarbamoylases were isolated as described by Nowlan and Kantrowitz (1985), from *E. coli* strain EK1104 [F$^-$ *ara, thi,* Δ*pro-lac,* Δ*pyrB, pyrF*$^{\pm}$, *rpsL*], containing the plasmid pEK2 (Smith et al., 1986) or pEK59, respectively. The plasmid pEK59 corresponds to the vector pUC119 and the wild-type *pyrBI* operon containing a small fragment carrying the desired mutation. For further details concerning the protocols used to construct plasmids like pEK59, see Ladjimi et al. (1987).

Determination of Protein Concentration. The concentration of pure wild-type holoenzyme was determined by absorbance measurements at 280 nm using an extinction coefficient of 0.59 cm^2/mg (Gerhart & Holoubek, 1967). The protein concentration of the mutant holoenzyme was determined by the Bio-Rad version of Bradford's dye-binding assay (Bradford, 1976).

Aspartate Transcarbamoylase Assay. The transcarbamoylase activity was measured at 25 °C by either a

[2] A suffix is appended to the residue number to distinguish the catalytic (c) and regulatory (r) chains of aspartate transcarbamoylase.

[3] The notation used to name the mutant enzymes is, for example, the Lys-60r → Ala enzyme. The wild-type amino acid and location within the regulatory (r) or catalytic (c) chains are indicated to the left of the arrow while the new amino acid is indicated to the right of the arrow.

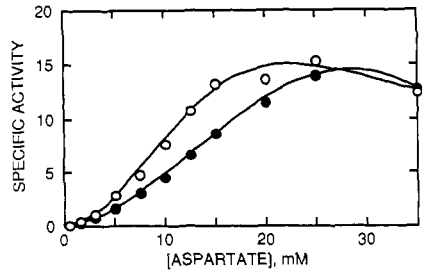

FIGURE 2: Aspartate saturating curves of the wild-type (●) and the Lys-60r → Ala (○) aspartate transcarbamoylases. Assays were performed at 25 °C in 0.05 M Tris–acetate buffer (pH 8.3) in the presence of saturating carbamoyl phosphate (19.2 mM). Specific activity is in units of millimoles per hour per milligram.

colorimetric (Pastra-Landis et al., 1981) or a pH-stat method (Wu & Hammes, 1973). pH-stat assays were carried out with a Radiometer TTT80 titrator and an ABU80 autoburet. All colorimetric assays were performed in duplicate, and the data points shown in the figures are the average.

Binding Measurements. The binding of CTP to the Lys-60r → Ala enzyme was determined by the technique of equilibrium dialysis using Spectra/Pro-2 (Spectrum Medical Industries) dialysis tubing. Dialysis experiments where carried out in microdialysis cells which hold 50 μL on each side of the dialysis membrane, which was pretreated as previously described (Jacobsberg et al., 1975). After equilibration for 18–20 h at 25 °C, 25-μL samples were removed from each side of the dialysis cell, and the concentration of CTP was determined by liquid scintillation employing a LKB 1217 Rackbeta liquid scintillation counter. Complete equilibration was confirmed under the experimental conditions. Equilibrium dialysis experiments were performed in 0.1 M imidazole/acetate buffer, 0.2 mM EDTA, and 2 mM 2-mercaptoethanol, pH 7, and the enzyme was dialyzed into this buffer before use.

For the dialysis experiments in which the competition between [^{3}H]ATP and CTP was investigated, the enzyme and [^{3}H]ATP concentrations were kept constant at 10–15 mg/mL and 1.43 mM (2.2×10^{-3} μCi/μL), respectively, on one side of the membrane while the concentration of CTP was varied on the other. Carbamoyl phosphate at an equilibrium concentration of 19.2 mM was also present in order to prevent the binding of the nucleotide effectors at the active site (Matsumoto & Hammes, 1973). The equilibrium competition experiments were performed under the same conditions as the CTP binding experiments described above.

Data Analysis. The analysis of the steady-state kinetic data was carried out as previously described by Silver et al. (1983). The analysis of the structural data, based on the three-dimensional coordinates of the enzyme with CTP bound (Kim et al., 1987), was accomplished by using the program PS300 FRODO (Department of Biochemistry, Rice University) on an Evans & Sutherland PS390 interfaced to a MicroVax Q5.

RESULTS

Construction of the Lys-60r → Ala Aspartate Transcarbamoylase by Site-Specific Mutagenesis. The replacement of lysine by alanine at position 60 in the regulatory chain of aspartate transcarbamoylase was accomplished by site-specific mutagenesis using the method of Zoller and Smith (1982), with modifications as previously described (Ladjimi & Kantrowitz, 1987).

Catalysis Is Not Altered by the Lys-60r → Ala Substitution. The introduction of alanine at position 60 in the regulatory chain of aspartate transcarbamoylase does not alter the maximal observed specific activity (14.4 mmol·h^{-1}·mg^{-1}) or

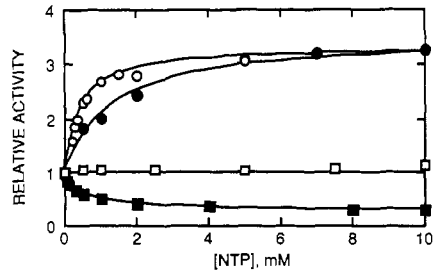

FIGURE 3: Influence of ATP and CTP concentrations on the activity of the wild-type and the Lys-60r → Ala aspartate transcarbamoylases. Assays were performed at 25 °C in 0.05 M Tris–acetate buffer (pH 8.3) in the absence or presence of increasing concentrations of either ATP or CTP (NTP). Carbamoyl phosphate concentration was saturating (19.2 mM), and aspartate concentration was held constant at the $[S]_{0.5}^{Asp}$ of the respective enzyme. Wild-type enzyme in the presence of ATP (●) and CTP (■); Lys-60r → Ala enzyme in the presence of ATP (○) and CTP (□).

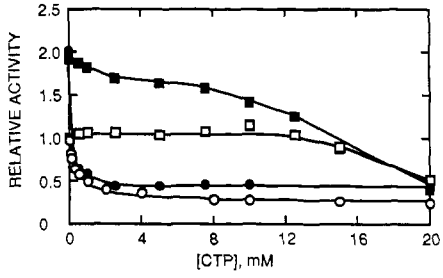

FIGURE 4: Effect of CTP concentration on the activity of the wild-type and Lys-60r → Ala aspartate transcarbamoylases in the absence and presence of ATP. Assays were performed at 25 °C in 0.05 M Tris–acetate buffer (pH 8.3). Carbamoyl phosphate concentration was saturating (19.2 mM), and the aspartate concentration was held constant at the $[S]_{0.5}^{Asp}$ of the respective enzyme. Wild-type enzyme in the absence of ATP (○) and in the presence of 0.5 mM ATP (●); Lys-60r → Ala enzyme in the absence of ATP (□) and in the presence of 0.5 mM ATP (■).

cooperativity (n_H = 1.8) of the enzyme as measured by the Hill coefficient (see Figure 2). However, the concentration of aspartate required to reach half the maximal observed specific activity ($[S]_{0.5}^{Asp}$) is reduced from 13.3 mM for the wild-type enzyme to 9.6 mM for the Lys-60r → Ala enzyme. Since the maximal velocity of the enzyme is not altered, the observed alteration in the $[S]_{0.5}^{Asp}$ suggests a slightly enhanced affinity of the mutant enzyme toward aspartate.

CTP No Longer Inhibits the Lys-60r → Ala Enzyme. In order to determine if ATP and CTP still influence the activity of the Lys-60r → Ala enzyme, saturation curves were determined for each of the nucleotides at each enzyme's respective $[S]_{0.5}^{Asp}$. In these experiments, high concentrations of carbamoyl phosphate (≈20 mM) were present to prevent the binding of the nucleotides at the active site. As seen in Figure 3, ATP activates the mutant enzyme 2.3-fold, almost identical with the effect that ATP has on the wild-type enzyme (2.5-fold). However, the concentration of ATP required to reach half of the maximal activation is substantially lower for the mutant (0.4 mM) than for the wild-type enzyme (1.2 mM), indicating that the affinity of the mutant enzyme for ATP is enhanced relative to that of the wild-type enzyme. The Lys-60r → Ala enzyme is not inhibited by CTP between 0 and 10 mM, although at higher concentrations of CTP, inhibition is observed (see Figure 4). However, at CTP concentrations higher than approximately 10 mM, inhibition at the active site cannot be completely distinguished from inhibition at the regulatory site.

In the Absence or Presence of CTP, UTP Does Not Inhibit the Lys-60r → Ala Enzyme. At pH 7.0, UTP has been found to enhance the CTP inhibition of aspartate transcarbamoylase

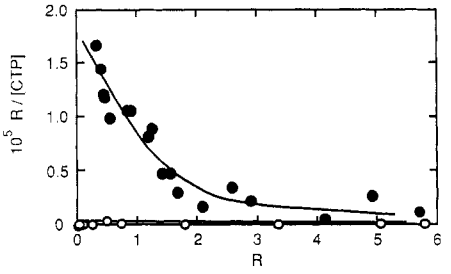

FIGURE 5: Binding of CTP to the wild-type (●) and the Lys-60r → Ala (○) aspartate transcarbamoylases. Equilibrium dialysis experiments were performed at 25 °C for 18–20 h in 0.1 M imidazole acetate buffer, 2 mM 2-mercaptoethanol, and 0.2 mM EDTA, pH 7.0. The binding data are represented as a Scatchard plot (Scatchard, 1949), R/[CTP] versus, R, where R is the number of moles of CTP bound per mole of aspartate transcarbamoylase (M_r 310 000) and [CTP] is the concentration of free CTP. The binding experiments were carried out in the presence of an equilibrium concentration of 19.2 mM carbamoyl phosphate to prevent CTP from binding at the active sites (Matsumoto & Hammes, 1973).

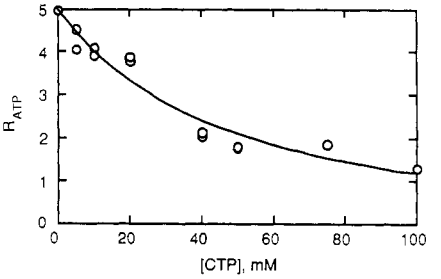

FIGURE 6: Competition between CTP and ATP for the nucleotide binding site. The data obtained from the equilibrium dialysis competition binding experiments show the decrease in R_{ATP} with increasing CTP concentrations for the Lys-60r → Ala enzyme (○), where R_{ATP} is the number of moles of ATP bound per mole of aspartate transcarbamoylase (M_r 310 000). Conditions are identical with those reported in Figure 5.

without being inhibitory itself in the absence of CTP (Wild et al., 1989). The Lys-60r → Ala enzyme was tested for UTP inhibition, under identical conditions used by Wild et al. (1989). The Lys-60r → Ala enzyme exhibits no detectable inhibition by UTP either in the absence or in the presence of 10 mM CTP (data not shown).

CTP Competes with ATP for the Regulatory Site of the Lys-60r → Ala Enzyme. In order to determine if CTP still can bind to the regulatory site of aspartate transcarbamoylase, a competition experiment was employed. The Lys-60r → Ala enzyme was activated by 0.5 mM ATP, which was sufficient to more than double the activity of the mutant enzyme; then the activity was measured as the CTP concentration was increased up to 20 mM. If CTP could still bind to the regulatory site of the enzyme, the ATP-enhanced activity of the enzyme would decrease as CTP replaced the bound ATP. For the wild-type enzyme, CTP will completely replace the bound ATP and reduce the activity of the enzyme to the same level as if CTP had been added alone (see Figure 4) (Changeux et al., 1968).

When increasing concentrations of CTP are added to the ATP-activated Lys-60r → Ala enzyme, the activation is completely reversed. For the wild-type enzyme, total reversal of the ATP activation occurs between 0.1 and 0.5 mM CTP, while the mutant enzyme requires more than 20 mM CTP to completely reverse the ATP activation (see Figure 4). Even though much more CTP is required to reverse the ATP activation of the Lys-60r → Ala enzyme compared to the wild-type enzyme, the reversal of the activation demonstrates that CTP can still bind and compete for the regulatory site of the mutant enzyme.

The Affinity of CTP for the Lys-60r → Ala Enzyme Is Reduced Substantially. In order to determine the effect of the Lys-60r → Ala substitution on the binding of CTP, equilibrium dialysis experiments were performed. As seen in Figure 5, the binding of CTP to the Lys-60r → Ala enzyme is reduced substantially; in fact, the binding of CTP is so low that neither an accurate dissociation constant nor the number of binding sites can be determined. From these experiments, the CTP dissociation constant (K_D^{CTP}) for the Lys-60r → Ala enzyme is estimated to be $>1 \times 10^{-3}$ M. This limit for the K_D^{CTP} is consistent with the kinetic competition experiments described above.

Since the affinity of CTP for the Lys-60r → Ala enzyme is so poor, a binding experiment was designed to more accurately determine the dissociation constant of CTP utilizing the fact that CTP and ATP compete for the regulatory site. By measuring the loss of bound ATP at increasing concentrations of CTP, the dissociation constants of both ATP and CTP to the Lys-60r → Ala enzyme could be estimated (Changeux et al., 1968). As seen in Figure 6, increasing concentrations of CTP result in decreased binding of ATP, and since CTP binding at the active site is not observed, substantially higher concentrations of CTP can be used in this experiment than in the kinetic competition experiments. Assuming there are six binding sites for ATP on the mutant enzyme, the dissociation constants for the binding of ATP and CTP to the Lys-60r → Ala enzyme are $(3.0 \pm 1.8) \times 10^{-4}$ and $(6.3 \pm 4.2) \times 10^{-3}$ M, respectively. Thus, the affinity of ATP to the Lys-60r → Ala enzyme increases by approximately 5-fold, and the affinity of CTP is reduced by approximately 100-fold compared to the affinity of these nucleotides for the wild-type enzyme.

Discussion

It has long been known that ATP and CTP bind to the same site on the allosteric domain of the regulatory chain of aspartate transcarbamoylase even though they have opposite effects on enzymatic activity (Changeux et al., 1968; Gerhart & Pardee, 1962, 1964; London & Schmidt, 1972, 1974). Analysis of the X-ray structure of the enzyme with CTP bound shows a large number of interactions between the ribose triphosphate portion of the nucleotides and the enzyme, but few specific interactions to the pyrimidine ring (Figure 1) (Kim et al., 1987). For example, Lys-94r interacts specifically with the triphosphate portion of the nucleotide, and the replacement of this side chain by glutamine resulted in loss of both ATP and CTP binding (Zhang et al., 1988). These results demonstrated conclusively that the triphosphate moiety of both ATP and CTP binds to the same subsite within the regulatory site as originally proposed by London and Schmidt (1972). One of few specific interactions between the enzyme and the pyrimidine ring of CTP is between the 2-keto group and Lys-60r, a side chain that also interacts with the 2′-OH of the ribose (see Figure 1). Since ATP does not have a keto group in the 2 position, we used site-specific mutagenesis to replace Lys-60r with alanine in order to determine if the interaction between Lys-60r and CTP is important for discrimination between the nucleotide effectors.

When Lys-60r is replaced by alanine, no alteration in the maximal observed specific activity is observed, which indicates that this amino acid alteration does not influence catalysis directly. However, this amino acid substitution does lower the $[S]_{0.5}^{Asp}$, suggesting that this regulatory site mutation, some 60 Å from the active site, can influence the affinity of the enzyme

for aspartate. The alteration in $[S]_{0.5}^{Asp}$ implies either that the T ⇌ R equilibrium has been altered by the substitution or that the substitution has caused conformation changes in the regulatory chain that can propagate across the C–R interface and can directly influence the affinity of the enzyme for aspartate at the active site. Since the $[S]_{0.5}^{Asp}$ for the mutant enzyme is reduced in an analogous fashion to that observed upon the addition of ATP to the wild-type enzyme, and the Lys-60r → Ala substitution results in increased affinity for ATP, it is tempting to speculate that the modification has shifted the conformation of the regulatory subunit toward the one that naturally occurs upon ATP binding, suggesting that Lys-60r in the wild-type enzyme may actually hinder the binding of ATP.

The replacement of Lys-60r by alanine does cause alterations in the response of the enzyme toward both ATP, CTP, and UTP. In the case of ATP, the mutant enzyme is still activated normally; however, the mutant enzyme responds to ATP at lower concentrations, indicating that ATP is binding more strongly to the Lys-60r → Ala enzyme than to the wild-type enzyme. At concentrations of CTP and UTP that normally inhibit the wild-type enzyme maximally, the Lys-60r → Ala enzyme is insensitive to either nucleotide. At high concentrations of CTP, some inhibition is observed; however, at concentrations of CTP above 10–20 mM, it is almost impossible to distinguish if CTP is inhibiting the enzyme at the regulatory site or is acting as a competitive inhibitor of carbamoyl phosphate at the active site. Binding measurements by equilibrium dialysis showed that the affinity of CTP for the Lys-60r → Ala enzyme is reduced approximately 100-fold. These data taken together suggest that Lys-60r is directly involved in the binding of CTP and is important for the discrimination between ATP and CTP. Although our data are not conclusive, they suggest that CTP can still inhibit the mutant enzyme and therefore Lys-60r may not be directly involved in the inhibition of the enzyme when CTP binds to the regulatory site.

UTP does not inhibit the Lys-60r → Ala enzyme either in the absence or in the presence of CTP under the conditions that were tested here. The analysis of the UTP experiments is complex for two reasons. First, since CTP binds so weakly to the mutant enzyme, there may not be sufficient CTP bound to UTP to have any effect. Second, since the 2-keto group is also present in UTP, the replacement of Lys-60r by alanine may have reduced the binding of UTP to such an extent that the normal UTP inhibition would not be observed under the conditions employed. A detailed description of the interaction of UTP with the Lys-60r → Ala enzyme must await the complete analysis of how UTP interacts and inhibits the wild-type enzyme in the presence of CTP, although it is tempting to speculate that Lys-60r is important for the binding of both the pyrimidine nucleotides but not for the binding of ATP. It we speculate that the binding of both CTP and UTP involves a link between Lys-60r and the 2-keto group on the pyrimidine ring, then the discrimination between these two nucleotides by the enzyme must be due to the group at the 4 position. In the case of CTP, this is an amino group, a hydrogen bond donor, and in the case of UTP, this is a keto group, a hydrogen bond acceptor. Therefore, a specific interaction between the enzyme and the 4 position of the pyrimidine ring may be crucial for the discrimination between CTP and UTP. In the X-ray structure of the enzyme with CTP bound, no specific interactions are observed between the enzyme and the amino group of CTP. However, the lack of a specific interaction may be due to the fact either that the enzyme has a hydrogen bond donor in this position for a specific interaction with only UTP or that the low pH used for the crystallization of the enzyme CTP complex may have resulted in an orientation of CTP which is slightly different from that observed under physiological conditions.

In the model proposed by London and Schmidt (1972) for nucleotide triphosphate binding to aspartate transcarbamoylase, the regulatory binding site is divided into two subsites, with one subsite binding the ribose triphosphate moiety and the other interacting with polar groups on the base. Differentiation between the purines and pyrimidines occurs exclusively at the latter subsite, since the remainder of the nucleotides are identical. These authors further propose that there must be specific interactions between the enzyme and the bases which allow for discrimination between the purines and pyrimidines. Since the Lys-60r → Ala enzyme is still weakly inhibited by CTP, these data suggest that Lys-60r in the wild-type enzyme is not fully responsible for the discrimination between the purine and pyrimidine nucleotides. However, these experiments with the Lys-60r → Ala enzyme have for the first time identified a residue which is directly involved in the binding of CTP, but actually hinders the binding of ATP. Additional experimental work is required to determine how CTP can inhibit and ATP can activate aspartate transcarbamoylase even through they both bind to the same site on the enzyme, and how they can alter enzymatic activity by binding to the regulatory site approximately 60 Å from the active site.

Acknowledgments

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Binding of Tissue-Type Plasminogen Activator to Lysine, Lysine Analogues, and Fibrin Fragments

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ABSTRACT: Human tissue-type plasminogen activator (t-PA) consists of five domains designated (starting from the N-terminus) finger, growth factor, kringle 1, kringle 2, and protease. The binding of t-PA to lysine–Sepharose and aminohexyl-Sepharose was found to require kringle 2. The affinity for binding the lysine derivatives 6-aminohexanoic acid and *N*-acetyllysine methyl ester was about equal, suggesting that t-PA does not prefer C-terminal lysine residues for binding. Intact t-PA and a variant consisting only of kringle 2 and protease domains were found to bind to fibrin fragment FCB-2, the very fragment that also binds plasminogen and acts as a stimulator of t-PA-catalyzed plasminogen activation. In both cases, binding could completely be inhibited by 6-aminohexanoic acid, pointing to the involvement of a lysine binding site in this interaction. Furthermore, the second site in t-PA involved in interaction with fibrin, presumably the finger, appears to interact with a part of fibrin, different from FCB-2.

Polymerized fibrin forms the network that keeps a blood clot together. After fibrin has fulfilled its function, it is degraded to soluble products by the relatively nonspecific serine protease plasmin. Plasmin is formed from its inactive zymogen plasminogen by plasminogen activators among which tissue-type plasminogen activator (t-PA)[1] has a prominent role. Plasminogen activation by t-PA is virtually confined to the fibrin surface by specific binding of t-PA to fibrin and the very low activity of t-PA that is not bound to fibrin (Rånby & Wallén, 1985; Thorsen et al., 1972; Hoylaerts et al., 1982). Upon binding to fibrin, plasminogen activation by t-PA is considerably accelerated. The formation of a ternary complex between fibrin, plasminogen, and t-PA appears to constitute the basis of the mechanism of acceleration of t-PA-catalyzed plasminogen activation by fibrin (Hoylaerts et al., 1982; Rånby, 1982). The involvement of lysine residues in fibrin in this stimulation was suggested by Radcliffe (1983). During the last few years, it has become clear that several enzymatically or chemically derived fragments of fibrin(ogen), such as D and FCB-2, retain a large part of the ability to accelerate t-PA-catalyzed plasminogen activation whereas other fragments, such as E, have no such effect (Verheijen et al., 1982a,b; Nieuwenhuizen et al., 1983a,b).

Fibrin fragment FCB-2, the most effective fragment for acceleration of t-PA, was shown to be able to bind t-PA and plasminogen and seems to be a good candidate for the fibrin site involved in formation of the ternary complex (Bosma et al., 1988). The involvement of lysine residue Aα 157 in this acceleration was shown in a series of elegant experiments using synthetic peptides (Voskuilen et al., 1987).

In the last few years, much knowledge has been gained concerning the structure and properties of t-PA. The cDNA

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[1] Abbreviations: t-PA, tissue-type plasminogen activator; mt-PA, melanoma cell derived t-PA; rt-PA, recombinant t-PA; ϵACA, 6-aminohexanoic acid; CHO, Chinese hamster ovary.

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