BIOCHEMISTRY
including biophysical chemistry & molecular biology

Rapid Report

pubs.acs.org/biochemistry

A Second Allosteric Site in *Escherichia coli* Aspartate Transcarbamoylase

Alexis W. Peterson, Gregory M. Cockrell, and Evan R. Kantrowitz*

Department of Chemistry, Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02467, United States

Supporting Information

ABSTRACT: *Escherichia coli* aspartate transcarbamoylase is feedback inhibited by CTP and UTP in the presence of CTP. Here, we show by X-ray crystallography that UTP binds to a unique site on each regulatory chain of the enzyme that is near but not overlapping with the known CTP site. These results bring into question all of the previously proposed mechanisms of allosteric regulation in aspartate transcarbamoylase.

Escherichia coli aspartate transcarbamoylase (EC 2.1.3.2, aspartate carbamoyltransferase, ATCase) is a model for allosteric regulation.[1] The enzyme catalyzes the committed step in pyrimidine nucleotide biosynthesis, the formation of *N*-carbamoyl-L-aspartate and phosphate from carbamoyl phosphate and L-aspartate.[2] *N*-Carbamoyl-L-aspartate then continues through the pathway to form UTP and CTP. ATCase is regulated homotropically by L-aspartate and heterotropically by nucleotide effectors. ATP, the end product of the purine biosynthetic pathway, is an activator of ATCase, while CTP, as one of the end products of the pyrimidine pathway, is an inhibitor. The pyrimidine nucleotide UTP does not inhibit the enzyme alone, but it synergistically inhibits the enzyme in the presence of CTP.[3]

E. coli ATCase is a dodecamer composed of six regulatory chains (M_r = 17000) and six catalytic chains (M_r = 33000), organized into two catalytic trimers and three regulatory dimers. The substrate binding sites, responsible for enzyme activity, are located in the catalytic chains, whereas the nucleotide effectors bind in the regulatory chains.[4] There are two states of the enzyme characterized by large changes in quaternary structure: the low-activity, low affinity for the substrate T state and the high-activity, high affinity for the substrate R state. The substrates and allosteric effectors influence the equilibrium between the T state and the R state structure.[5]

Structural and kinetic analyses have shown that ATP and CTP, which exhibit opposing effects on ATCase activity, compete for and bind to the same sites on the regulatory chains,[6] with one binding site per chain. Similarly, other studies have indicated that UTP and ATP bind competitively.[7] Binding experiments have shown that for both CTP and ATP there are three high-affinity sites and three low-affinity sites per holoenzyme,[8] suggesting that each regulatory dimer contains one high-affinity site and one low-affinity site. Binding experiments using the isolated regulatory dimer confirm the two classes of sites with high and low affinities.[8] To explain the synergistic inhibition by UTP in the presence of CTP, it has been proposed that CTP binds to the high-affinity sites while UTP binds to the low-affinity sites, because addition of UTP to ATCase with CTP bound results in a decrease in the number of CTP binding sites from six to three.[9]

The binding of nucleotides to the regulatory sites was further investigated using an unnatural fluorescent amino acid [L-(7-hydroxycoumarin-4-yl)ethylglycine] in place of Glu52, located near the nucleotide binding site.[10] Fluorescence measurements showed that CTP and UTP do not bind competitively, consistent with the notion of a functional asymmetry between the two allosteric sites of the regulatory dimer. This study also indicated that ATP, CTP, GTP, and UTP bind with similar affinities and have two classes of binding sites, one with high affinity and one with low affinity. However, other studies have shown that UTP has a much lower binding affinity than CTP.[11−13]

While CTP and UTP are structurally very similar, they have very different effects on ATCase activity. To further elucidate the mechanism of UTP action, specifically the synergistic inhibition in the presence of CTP and the lack of inhibition by UTP alone, X-ray crystallography was used here to determine the structure of ATCase in the presence of UTP. R state crystals of ATCase were obtained by microdialysis in 50 μL wells using previously reported conditions.[14] The crystals were then dialyzed overnight in crystallization buffer with 10 mM UTP. Before being flash-frozen in liquid nitrogen, the crystals were dipped into UTP crystallization buffer with 20% (v/v) 2-methyl-2,4-pentanediol. These crystals diffracted to a maximal resolution of 2.3 Å in space group *P*321 (Table S1 of the Supporting Information). The 2.1 Å R state structure of ATCase [Protein Data Bank (PDB) entry 1D09][15] was used as the initial model. After a number of rounds of refinement, model building, and water placement, the final R_{factor} and R_{free} values were 0.173 and 0.207, respectively. As an indication of the quality of the refinement, Figure S1 of the Supporting Information shows a portion of the electron density map corresponding to a portion of the regulatory chain. Residues 1−10 of the regulatory chains were omitted from the final model, because of weak electron density. Complete experimental details, including data collection and refinement, are provided in the Supporting Information. Coordinates and structure factors for the structure have been deposited as PDB entry 4F04.

Received: May 14, 2012
Revised: June 4, 2012
Published: June 5, 2012

dx.doi.org/10.1021/bi3006219 | *Biochemistry* 2012, 51, 4776−4778

The main-chain root-mean-square deviation (rmsd) between the 1D09 and UTP-bound structures was 0.42 Å, indicating that the binding of UTP had little overall influence on the structure of the enzyme. For the regulatory chain and its allosteric domain, the main-chain rmsds between the structure with and without UTP were 0.43 and 0.47 Å, respectively, suggesting that UTP binding may result in localized conformational changes near its binding site. Figure 1 shows a

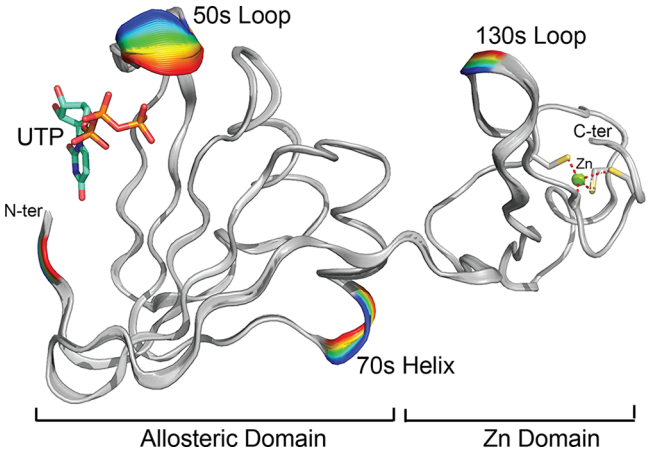

Figure 1. Structural changes upon the binding of UTP to a regulatory chain of ATCase. The structures of ATCase in the absence and presence of UTP (PDB entry 1D09) are shown with blue and red highlights, respectively. Thirty structures are shown, calculated linearly between the two determined X-ray structures. The portions of the structures with a main-chain rmsd of >1.5 Å are spectrally colored.

representation of the conformational changes that occur in the r6 regulatory chain due to the binding of UTP. Besides motions near the N- and C-termini, the major conformational change is restricted to the 50s loop (residues 48−58), with smaller motions in the 70s helix (residues 68−77) and the 130s loop (residues 129−134).

The binding site of UTP is located adjacent to, but not overlapping with, the site canonically occupied by CTP and ATP.[6] In the *P*321 space group, the asymmetric unit is composed of chains c1, r1, r6, c6, or one-third of the holoenzyme. The electron density for UTP is not as strong as the density for the protein overall. In addition, the electron density for UTP is weaker in the r1 chain than in the r6 chain. Still, the data clearly show that UTP binds in this unique location on the regulatory chain.

The relatively weak electron density observed for UTP is reflected in the high *B* factors of the UTP atoms, 173 and 140 Å^2 for UTP in the r1 and r6 chains, respectively. These values are considerably higher than the *B* factors for the atoms of the r1 (76 Å^2) and r6 (94 Å^2) chains. The weaker electron density of UTP in the r1 versus r6 regulatory chains suggests that these two sites have different affinities for UTP. This supposition is supported by the data, which show that the UTP in the r6 chain forms more interactions with the protein than the UTP in the r1 chain. Figure 2 shows the interactions between UTP and the r6 chain, and stereoviews of UTP bound in the r1 and r6 chains are shown in Figure S2 of the Supporting Information. Of particular note is the stretch of residues Leu48, Pro49, Ser50, Gly51, Glu52, Lys56, Leu58, and Lys60, which all interact with UTP in the r6 chain. These residues comprise the 50s loop that undergoes the largest structural alteration upon UTP binding

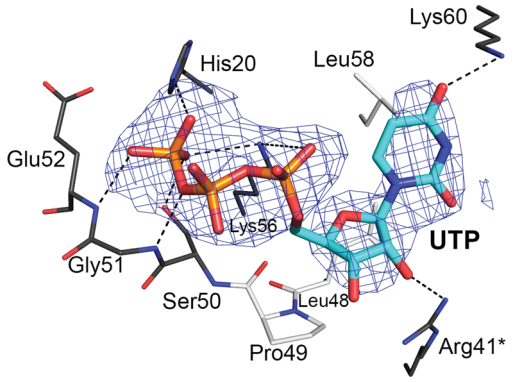

Figure 2. UTP bound to the r6 regulatory chain of ATCase. Residues making polar interactions (---) are shown with gray carbons, while those making hydrophobic interactions are shown with white carbons. The asterisk after Arg41 indicates that it is donated from the r1 chain.

(see Figure 1). In addition to these residues, Glu10, His20, and Arg41 also interact with UTP. The interaction between UTP and Arg41 is between UTP bound to the r6 chain and Arg41 of the r1 chain. A complete list of the interactions with UTP in the r1 and r6 chains is provided in Table S2 of the Supporting Information. The smaller number of interactions between residues in the r1 chain and UTP reinforces the asymmetric nature of the r1 and r6 chains and provides support for the previously proposed hypothesis that there are two classes of UTP binding sites having different affinities for the nucleotide.[10]

To show the spatial relationship between the new nucleotide-binding site and the canonical site, the surfaces of the regulatory chain with CTP (PDB entry 8AT1)[16] and UTP bound are shown in panels a and b of Figure 3, respectively. To

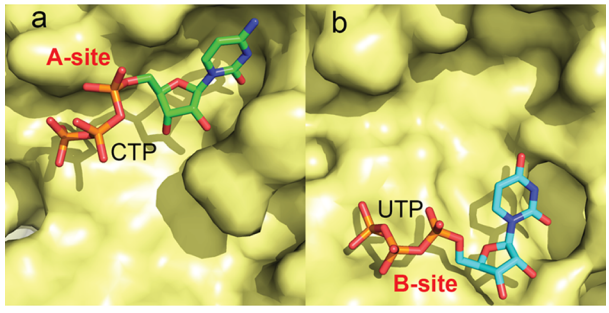

Figure 3. Comparison of the A and B nucleotide-binding sites using PDB entry 8AT1 to show the CTP binding location. The identical orientation is shown for (a) CTP (green carbons) bound in the A site and (b) UTP (teal carbons) bound in the B site.

distinguish between the two nucleotide binding sites in each regulatory chain, the canonical site where ATP and CTP bind will be called the A site while the newly discovered, UTP-binding site will be termed the B site. Thus, each of the six regulatory chains of ATCase has two, A and B, nucleotide sites.

Some of the residues that make direct interactions with UTP have been previously studied by site-specific mutagenesis. The H20A[17] or K56A[18] mutation resulted in the complete loss of synergistic inhibition by UTP. These two residues make direct contacts with UTP in the B site, as shown in Figure 2, explaining the loss of synergistic inhibition. In the case of the K56A mutant, previous studies could not explain the loss of

dx.doi.org/10.1021/bi3006219 | *Biochemistry* 2012, 51, 4776−4778

synergistic inhibition as Lys56 did not show any interactions with CTP.

The X-ray structure reported here shows that UTP binds to the B site, a site different from that normally occupied by CTP or ATP (A site). The lack of any evidence of binding of UTP in the A site suggests that UTP has a significantly higher affinity for the B site than the A site. The difference in UTP affinity between the A and B sites can be explained by electrostatic interactions between the UTP and Lys60. The 4-keto group of UTP has a more favorable electrostatic interaction with the ε-amino group of Lys60 than with the main-chain oxygen atoms of Ile12 and Tyr89 in the A site. The oxygen atoms of Ile12 and Tyr89 as well as the 4-keto group of UTP bear partial negative charges because of the resonance, leading to charge repulsion between the protein and UTP in the A site. This also explains why previous crystallographic studies have not observed CTP in the B site because positive charge−charge repulsion would occur between the ε-amino group of Lys60 and the 4-amino group of CTP.

With the discovery that UTP binds to a unique site on the regulatory chain, we propose a revised mechanism of UTP action that is consistent with the structural data. The X-ray structure of ATCase in the presence of UTP reported here provides an explanation for the lack of inhibition of the enzyme by UTP alone, and the ability of UTP to synergistically inhibit ATCase in the presence of CTP. As seen in panels a and b of Figure 3, the binding sites of CTP and UTP on one regulatory chain of ATCase are in the proximity of each other but do not overlap. When UTP alone is present, binding occurs exclusively at the B site, because of the stabilizing interactions between Lys60 and the 4-keto group of uracil mentioned above. This same interaction prevents or weakens binding of CTP at the B site. This is supported by the fact that even at relatively high concentrations of CTP, no CTP is observed in the B site by X-ray crystallography.[16] However, when UTP binds to the B site no allosteric inhibition is observed.

When CTP is present in the A site (see Figure 3a), there is sufficient space for UTP to bind to the B site. Previous studies have indicated that the affinity of CTP is enhanced in the presence of UTP, and the binding of UTP is enhanced upon the binding of CTP.[11] These data suggest that the two nucleotides act together in the combined AB allosteric site to synergistically enhance the binding of the other and induce conformational changes that shift the T to R equilibrium further toward the T state than CTP alone can induce. Thus, the binding of UTP to the B site functions to amplify the effect of the nucleotide bound in the A site. The molecular basis of the synergistic inhibition could be via interactions with the 50s loop, the N-terminus, and/or the r1−r6 interface, all of which are close to the B site.

The structure of ATCase in the presence of UTP reported here shows that the previously hypothesized CTP−UTP binding pattern is incorrect. It agrees with the notion that there is a functional asymmetry between the nucleotide sites of a regulatory dimer and indicates that there are four distinct nucleotide binding sites per dimer. With the discovery of this new allosteric site in *E. coli* ATCase, the previously proposed mechanisms for allosteric regulation based on two asymmetric nucleotide binding sites per regulatory dimer must be revised. More crystallographic studies are needed to determine conclusively the mechanism of the synergistic effect, although this study provides insights into the likely binding pattern.

■ ASSOCIATED CONTENT

Supporting Information

Experimental material and methods, Tables S1 and S2, and Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: evan.kantrowitz@bc.edu. Telephone: (617) 552-4558.

Funding

This work was supported by Grant GM26237 from the National Institutes of Heath. Use of the National Synchrotron Light Source, Brookhaven National Laboratory, was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract DE-AC02-98CH10886.

Notes

The authors declare no competing financial interests.

■ ACKNOWLEDGMENTS

We thank Howard Robinson of the Brookhaven National Laboratory for data collection and assistance with data processing.

■ REFERENCES

(1) Kantrowitz, E. R. (2012) *Arch. Biochem. Biophys. 519*, 81−90.
(2) Jones, M. E., Spector, L., and Lipmann, F. (1955) *J. Am. Chem. Soc. 77*, 819−820.
(3) Wild, J. R., Loughrey-Chen, S. J., and Corder, T. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 46−50.
(4) Gerhart, J. C., and Schachman, H. K. (1965) *Biochemistry 4*, 1054−1062.
(5) Howlett, G. J., Blackburn, M. N., Compton, J. G., and Schachman, H. K. (1977) *Biochemistry 16*, 5091−5099.
(6) Stevens, R. C., Gouaux, J. E., and Lipscomb, W. N. (1990) *Biochemistry 29*, 7691−7701.
(7) Changeux, J., Gerhart, J., and Schachman, H. (1968) *Biochemistry 7*, 531−538.
(8) Suter, P., and Rosenbusch, J. P. (1977) *J. Biol. Chem. 252*, 8136−8141.
(9) Fetler, L., Tauc, P., Herve, G., Cunin, R., and Brochon, J. C. (2001) *Biochemistry 40*, 8773−8782.
(10) Mendes, K. R., Martinez, J. A., and Kantrowitz, E. R. (2010) *ACS Chem. Biol. 5*, 499−506.
(11) England, P., and Hervé, G. (1992) *Biochemistry 31*, 9725−9732.
(12) Zhang, Y., and Kantrowitz, E. R. (1991) *J. Biol. Chem. 266*, 22154−22158.
(13) England, P., and Hervé, G. (1994) *Biochemistry 33*, 3913−3918.
(14) Stevens, R. C., Reinisch, K. M., and Lipscomb, W. N. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6087−6091.
(15) Jin, L., Stec, B., Lipscomb, W. N., and Kantrowitz, E. R. (1999) *Proteins: Struct., Funct., Genet. 37*, 729−742.
(16) Gouaux, J. E., Stevens, R. C., and Lipscomb, W. N. (1990) *Biochemistry 29*, 7702−7715.
(17) Zhang, Y., and Kantrowitz, E. R. (1992) *Biochemistry 31*, 792−798.
(18) Corder, T. S., and Wild, J. R. (1989) *J. Biol. Chem. 264*, 7425−7430.