# Mechanism of Adenylate Kinase. 20. Probing the Importance of the Aromaticity in Tyrosine-95 and the Ring Size in Proline-17 with Unnatural Amino Acids

Zhong Zhao,[†] Xiaohong Liu,[‡] Zhengtao Shi,[†] Lora Danley,[⊥] Baohua Huang,[‡] Ru-Tai Jiang,[†] and Ming-Daw Tsai*[,†,‡,§]

*Departments of Chemistry and Biochemistry and Ohio State Biochemistry Program The Ohio State University, Columbus, Ohio 43210 Department of Chemistry, University of California at Berkley, Berkley, California 94720*

*Received January 11, 1996*

We report an application of the unnatural amino acid mutagenesis developed by Schultz[1] to probe the importance of the aromaticity of tyrosine-95 and the ring size of proline-17 in the function of adenylate kinase (AK, from chicken muscle, overexpressed in *Escherichia coli*).[2]

AK catalyzes the reaction MgATP + AMP ⇌ MgADP + ADP. On the basis of structural analyses by X-ray[3] and NMR,[4] Tyr-95 is located in proximity to the adenosine moiety of AMP (within the range for amino−aromatic interaction, a weakly polar interaction[5]) and is also likely to be involved in aromatic−aromatic interactions[5a] with Phe-12 and Phe-105 (distances between centroids are 6.1 and 5.9 Å, respectively[3c]). A stereoview of the structure of *E. coli* AK complexed with AMP and AMPPNP (adenosine 5′-[$\beta,\gamma$-imido]triphosphate) is shown in Figure 1.[3a] The aromaticity of residue 95 is absolutely conserved; while it is Tyr in muscle AK, it is Phe in yeast and *E. coli* AK. Replacement of the Tyr-95 of muscle AK with Phe led to no detectable changes,[6a] while replacement with nonaromatic residues in both muscle and *E. coli* AK led to large decreases in activity.[6b,c] Since natural nonaromatic amino acids are very different from Tyr or Phe in the side chain structure, we used 2,5-dihydrophenylalanine (DiHPhe, Figure 2) to probe the importance of aromaticity in Tyr-95. DiHPhe can provide $\pi$-electrons without aromaticity, and its ring is close to planar.[7]

Proline-17 (Figure 1) is absolutely conserved in the phosphate binding loop (P-loop, GXPGXGKGT) in the AK family.[8]


**Figure 1.** Stereoview of the structure of *E. coli* AK complexed with AMP and AMPPNP.[3a] Pro-9 and Phe-86 corresopnd to Pro-17 and Tyr-95, respectively, in muscle AK.

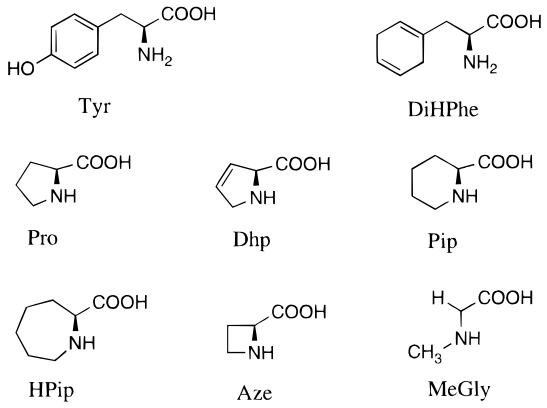


**Figure 2.** Structures of amino acids substituted for Tyr-95 and Pro-17. Tyr (tyrosine); DiHPhe (2,5-dihydrophenylalanine); Pro (proline); Dhp (3,4-dehydroproline); Pip (pipecolic acid); HPip (homopipecolic acid); Aze (azetidine 2-carboxylic acid); MeGly (*N*-methyl glycine).

Substitutions of Pro-17 with natural amino acids caused perturbations in substrate binding parameters.[9] However, natural amino acids cannot probe the importance of ring size, which is the key feature of proline. We used four proline analogs with different ring sizes (Figure 2), pipecolic acid (Pip), homopipecolic acid (HPip), 3,4-dehydroproline (Dhp), and azetidine 2-carboxylic acid (Aze), to probe the importance of the ring size of Pro-17 in the function of AK.

The unnatural amino acids were purchased or synthesized according to known procedures.[10] The suppressor tRNA aminoacylated with unnatural amino acids was prepared according to the procedures of Schultz.[1] To facilitate the purification of the *in vitro* synthesized AK, a six-histidine tag[11] was attached to the C-terminus of AK by modifying the gene of AK. The AK with the six-His tag (AKH) was first expressed in *E. coli*, purified, and shown to behave essentially the same as wild type (WT) AK. The AKH gene was then cloned into a high-copy-number expression vector PUK constructed in our lab.[12] *In vitro* protein syntheses were carried out with the coupled transcription/translation system of *E. coli* developed by Zubay[13a] with some modifications by Collins,[13b] Pratt,[13c] and Schultz.[1] *In vitro* expression of the wild type AKH gene under the control of a *tac* promoter in the PUK vector afforded ca. 5

* Address correspondences to: Department of Chemistry, The Ohio State University, 100 West 18th Ave., Columbus, OH 43210-1173.
[†] Department of Chemistry.
[‡] Ohio State Biochemistry Program.
[§] Department of Biochemistry.
[⊥] University of California at Berkley.

(1) (a) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, 244, 182. (b) Ellman, J. A.; Mendel, D.; Anthony-Cahill, S. J.; Noren, C. J.; Schultz, P. G. *Methods Enzymol.* **1991**, *202*, 301. (c) Roberson, S. A.; Ellman, J. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 2722. (d) Cornish, V. W.; Mendal, D.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 621.
(2) Tsai, M.-D.; Yan, H. *Biochemistry* **1991**, *30*, 6806.
(3) (a) Berry, M. B.; Meador, B.; Bilderback, T.; Liang, P.; Glaser, M.; Phillips, G. N., Jr. *Proteins* **1994**, *19*, 183. (b) Müller, C. W.; Schultz, G. E. *J. Mol. Biol.* **1992**, *224*, 159. (c) Dreusicke, D.; Karplus, P. A.; Schultz, G. E. *J. Mol. Biol.* **1988**, *199*, 359.
(4) Byeon, I.-J. L.; Yan, H.; Edison, A. S.; Mooberry, E. S.; Abildgarrd, F.; Markley, J. L.; Tsai, M.-D. *Biochemistry* **1993**, *32*, 12508.
(5) (a) Burley, S. K.; Petsko, G. A. *Adv. Protein Chem.* **1988**, *39*, 125. (b) Burley, S. K.; Petsko, G. A. *FEBS Lett.* **1986**, *203*, 139. (c) Deakyne, C. A.; Moet-Ner (Mautner), M. *J. Am. Chem. Soc.* **1985**, *107*, 474.
(6) (a) Kim, H. J.; Nishikawa, S.; Tanaka, T.; Uesugi, S.; Takenaka, H.; Hamada, M.; Kuby, S. A. *Protein Eng.* **1989**, *2*, 379. (b) Liang, P.; Phillips, G. N., Jr.; Glaser, M. *Proteins* **1991**, *9*, 28. (c) Tian, G.; Tsai, M.-D. Unpublished result.
(7) (a) Hermes, J. D.; Weiss, P. M.; Cleland, W. W. *Biochemistry* **1985**, *24*, 2959. (b) Snow, M. L.; Lauinger, C.; Ressler, C. *J. Org. Chem.* **1968**, *33*, 1774. (c) Hanson, K. R.; Havir, E. A.; Ressler, C. *Biochemistry* **1979**, *18*, 1431.
(8) (a) Egner, U.; Tomasselli, A. G.; Schultz, G. E. *J. Mol. Biol.* **1987**, *195*, 649. (b) Dreusicke, D.; Karplus, P. A.; Schultz, G. E. *J. Mol. Biol.* **1988**, *199*, 359. (c) Saraste, M.; Sibbald, P. R.; Wittinghofer, A. *TIBS* **1990**, *15*, 430.
(9) (a) Reinstein, J.; Brune, M.; Wittinghofer, A. *Biochemistry* **1988**, *27*, 4712. (b) Tagaya, M.; Yagami, T.; Noumi, T.; Futai, M.; Kishi, F.; Nakazawa, A.; Fukui, T. *J. Biol. Chem.* **1989**, *264*, 990.
(10) All amino acids used were of L configuration. Pip, Aze, Dhp, and MeGly were commercially available. DiHPhe was synthesized from Phe by Birch reduction as in ref 7b. HPip was initially provided by D. Seebach and J. Podlech at the Swiss Federal Institute of Technology at Zürich and then synthesized according to Seebach, D.; Dziadulewicz, E.; Berhrendt, L.; Cantoreggi, S.; Fitzi, R. *Liebigs Ann. Chem.* **1989**, 1215.
(11) (a) Arnold, F. H. *Bio/Technology* **1991**, *9*, 151. (b) Hoffmann, A.; Roeder, R. G. *Nucleic Acids Res.* **1991**, *19*, 6337.
(12) Huang, B.; Shi, Z.; Tsai, M.-D. *Gene* **1994**, *151*, 143.

0002-7863/96/1518-3535$12.00/0 

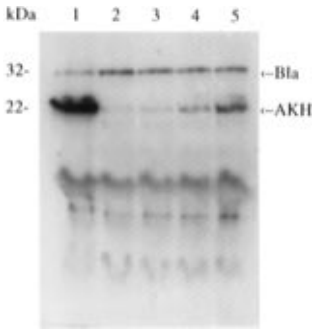


**Figure 3.** Autoradiograms of *in vitro* pipecolic acid suppression in the presence of [$^{35}$S]-L-methionine. Lane 1: expression of WT AKH; Lanes 2−5: suppressions with pipcolyl suppressor tRNA, with [$Mg^{2+}$] = 6.0, 8.0, 10.0, and 12.0 mM, respectively. The samples were centrifuged, and the supernatants were analyzed by 0.1% SDS−15% PAGE. Bla: $\beta$-lactamase.

**Table 1.** Summary of Suppression Efficiencies and Kinetic Data[a]

| protein | suppression efficiency (%)[b] | $K_{m,AMP}$ (mM) | $K_{m,MgATP}$ (mM) | $k_{cat}$ ($s^{-1}$) |
|---|---|---|---|---|
| WT AKH | 100 | 0.11 | 0.019 | 420 |
| Tyr95DiHPhe | nd | 0.54 | nd | 520 |
| Pro17Dhp | nd | 0.30 | 0.14 | 410 |
| Pro17Pip | 20 | 0.060 | 0.037 | 500 |
| Pro17HPip | 12 | 0.059 | 0.039 | 200 |
| Pro17Aze | 17 | nd | nd | <4.2[c] |

[a] The kinetic data should be considered as apparent values since they were obtained by holding one substrate constant while varying the other, instead of varying both substrates. The AMP concentration was held at 2.0 mM while [ATP] was varied, and [ATP] was held at 2.0 mM while [AMP] was varied; [$Mg^{2+}$] was kept at 4.8 mM. The abbreviation nd means not determined. [b] The suppression efficiency was measured by comparing the radioactivity of the band with that of *in vitro* expressed WT AKH. [c] The activity of this mutant was too low to detect and was estimated to be <1% of the $k_{cat}$ of WT.

$\mu$g of AK/mL, which was then purified by $Ni^{2+}$-affinity chromatography on a His-Bind resin column in a yield of ca. 35%. Incorporation of unnatural amino acids into AKH was accomplished by *in vitro* suppression of a TAG amber mutant using the chemically aminoacylated suppressor tRNA.[1] The suppression efficiency was optimized by varying [$Mg^{2+}$], as shown in Figure 3 for Pro17Pip. The suppression efficiency of various mutants are shown in Table 1. In addition to the four ring analogs, substitution of Pro-17 with *N*-methylglycine (MeGly) was also attempted, but the suppression efficiency was too low.

The purified AKH and mutants were subjected to steady-state kinetic analyses.[14] The results in Table 1 indicate that the Tyr95DiHPhe mutant behaves very similarly to WT. The $k_{cat}$ is virtually unchanged while the $K_{m,AMP}$ shows a small, 5-fold increase. The results suggest that the aromaticity of Tyr-95 is not critically important for the AK−AMP interactions. While replacements with natural nonaromatic amino acids caused large decreases in $k_{cat}$,[6b] the more isosteric DiHPhe is a reasonably good mimic of tyrosine or phenylalanine at position 95. The aromatic−aromatic interactions between Tyr-95 and other aromatic residues (Phe-12 and 105) are not expected to play important structural roles since Phe-12 and Phe-105 are not conserved in yeast and *E. coli* AK. Structural perturbation in the mutant, if any, should not be severe enough to perturb the function.

Replacements of Pro-17 with Dhp, Pip, and HPip resulted in little changes in kinetic parameters, except a 7-fold increase in the $K_{m,MgATP}$ of Pro17Dhp. The only mutant displaying a large decrease in activity is Pro17Aze, whose activity is beyond the limit of detection (<1% of WT's activity). In the absence of structural analysis, one cannot rule out the possibility that the functional perturbation of Pro17Aze was caused by global structural changes. However, a possible interpretation of the results is that the reduced activity of Pro17Aze is caused by the restricted conformational freedom of the four-membered ring of Aze.[15] It could restrict the movement of the P-loop and impair the conformational changes obligatory for the catalysis by AK. Molecular modeling and nuclear magnetic resonance studies should provide additional information.

In summary, we have used unnatural amino acid mutagenesis to show that the aromaticity of Tyr-95 (or Phe-95) is not critically important for the function of AK and that the P-loop of AK can tolerate replacement of Pro-17 with more flexible analogs, but not the more rigid four-membered ring analog Aze. To our knowledge, only one of the analogs in Table 1 (pipecolic acid) has previously been incorporated into other proteins.[16]

**Acknowledgment.** This work was supported by research Grant GM43268 from NIH. For paper 19 in the series, see ref 17. The first three authors contributed approximately equally to this work. The authors are greatly indebted to P. G. Schultz of the University of California at Berkeley, who helped us to initiate the project by hosting one of us (Z.S.) in their lab for one month in 1992 and by providing technical advice subsequently. We are grateful to D. Seebach for the generous gift of homopipecolic acid.

JA9600901

(13) (a) Zubay, G. *Annu. Rev. Genet*. **1973**, *7*, 267. (b) Collins, J. *Gene*, **1979**, *6*, 29. (c) Pratt, J. M. In *Transcription and Translation*; Hanes, B. D., Higgins, S. J., Eds.; IRL Press: Oxford, 1984; pp 179−209.

(14) Tian, G.; Sanders, C. R., II; Kishi, F.; Nakazawa, A.; Tsai, M.-D. *Biochemistry* **1988**, *27*, 5544.

(15) (a) Cesari, M.; D'llario, L.; Goglio, E.; Perego, G. *Acta Crystallogr., Sect. B* **1975**, *31*, 49. (b) Tsai, F.-H.; Overberger, C. G.; Zand, R. *Biopolymer* **1990**, *30*, 1039.

(16) (a) Ellman, J. A.; Mendal, D.; Schultz, P. G. *Science* **1992**, *255*, 197. (b) Chung, H.-H.; Benson, D. R.; Schultz, P. G. *Science* **1993**, *259*, 806. (c) Chung, H.-H.; Benson, D. R.; Cornish, V. W.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10145.

(17) Beichner, S.; Byeon, I.-J. L.; Tsai, M.-D. In *Peptides: Chemistry, Structure, and Biology*; Kaumaya, P. T. P., Hodges, R. S., Eds.; Escom: Leiden, 1996; pp 721−723.