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

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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′-[β,γ-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 π-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.

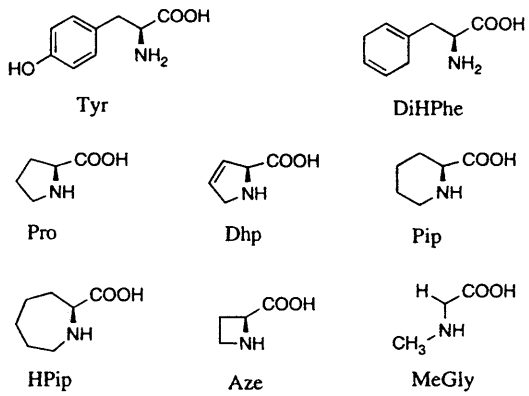

Figure 2. Structures of amino acids substituted for Tyr-95 and Pro-17. Tyr (tyrosine); DiHPhe (2,5-dihydrophenylalanine); Pro (proline); Dhp (3,4-dehydroproline); Pip (pipecolic acid); HPip (homopipecolic acid); Aze (azetidine 2-carboxylic acid); MeGly (*N*-methyl glycine).

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

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(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.

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