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Site-directed mutagenesis of AMP-binding residues in adenylate kinase

Alteration of substrate specificity

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Adenylate kinase is highly specific for AMP as phosphoryl acceptor. We have found that the replacement of Thr[39] by Ala in the chicken muscle enzyme, alone or together with the replacement of Leu[66] by Ile, caused remarkable increases in CMP and UMP activities with a concomitant decrease in AMP activity; therefore, the resulting mutant enzymes show CMP and UMP activities/AMP activity ratios much higher than the wild-type enzyme. The mutant enzyme in which Ala is substituted for Thr[39] has a V_{max} value for CMP comparable to that of CMP-UMP kinase.

Adenylate kinase; UMP-CMP kinase; Site-directed mutagenesis; Substrate specificity; AMP-binding

1. INTRODUCTION

Adenylate kinase (EC 2.7.4.3) catalyzes the reversible phosphoryl transfer between adenine nucleotides:

Mg-ATP + AMP ⇌ Mg-ADP + ADP.

The enzyme is highly specific for AMP as phosphoryl acceptor; only dAMP, CMP, and UMP act as poor substrates (see Table I). Our recent mutagenesis studies showed that Leu[66], Val[67], and Gln[101] in the chicken muscle enzyme contribute to AMP binding through hydrophobic interactions and hydrogen-bonding [1,2]. Interestingly, the replacement of Gln[101] by Met or His markedly increased the enzymatic activity for UMP [2].

The amino acid sequences of adenylate kinases from various sources show considerable similarities not only to each other but also to those of UMP-CMP kinases (EC 2.7.4.14) from yeast [3] and slime mold [4]. We have considered that the difference in substrate specificities between two different kinases is at least partly produced from the substitution of the amino acid residues located at the nucleoside monophosphate-binding site. Actually, Thr[39] and Leu[66] which are conserved in many adenylate kinases [5] and located close to the adenine ring of bound AMP [6] are replaced by Ala and Ile, respectively, in UMP-CMP kinases.

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Abbreviations: PCR, polymerase chain reaction; T39A and L66I, the single mutant enzymes in which Thr[39] and Leu[66] are separately replaced by Ala and Ile, respectively; T39A/L66I, the double mutant enzyme in which Thr[39] and Leu[66] are concomitantly replaced by Ala and Ile, respectively.

This paper reports the nucleoside monophosphate specificities of single and double mutant adenylate kinases for Thr[39] and Leu[66], as well as the results of kinetic analysis of those mutant enzymes.

2. EXPERIMENTAL

The replacements of Thr[39] and Leu[66] by other amino acids were accomplished by oligonucleotide-directed mutagenesis of the *Eco*RI-*Hin*dIII fragment (encoding chicken adenylate kinase) excised from the pKK-cAK1-1 [7] (kindly provided by Dr. A. Nakazawa of Yamaguchi University Medical School) and subcloned into the M13tv19 vector (Takara Shuzo), using synthetic six different oligonucleotide primers in combination, essentially as described previously [1]. The mutated enzyme genes were sequenced to confirm their structures. The 0.7-kbp fragment was inserted into plasmid pKK223-3 (Pharmacia), and transformed into *E. coli* JM109 cells. The mutant enzymes were purified to homogeneity, as previously described for the wild-type enzyme [8]. CD spectra in a region of 200–320 nm of all the mutant enzymes coincided well with that of the wild-type enzyme, suggesting the absence of gross structure changes.

The enzyme was assayed in the standard assay mixture containing 87 mM triethanolamine-HCl (pH 7.0), 10 mM $MgCl_2$, 100 mM KCl, 1 mM phosphoenolpyruvate, 0.16 mM NADH, 5 units of pyruvate kinase, and 20 units of lactate dehydrogenase, as described previously [1]. One unit of enzyme activity is defined as the amount of enzyme that produces 1 μmol of ADP per minute. When nucleoside monophosphates other than AMP were used as substrate, 20 units of pyruvate kinase were included. Steady state kinetic parameters were measured by a systematic variation in concentrations of AMP, CMP, or UMP as phosphoryl acceptor, with Mg-ATP as donor [1].

3. RESULTS

Table I compares the specific activities of the mutant enzymes for AMP, dAMP, CMP, and UMP with those of the wild-type enzyme. (GMP and IMP did not serve as acceptor for both wild-type and mutant enzymes.)

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Little decrease in AMP activity was observed for T39A, whereas considerable decreases appeared for L66I and T39A/L66I. Similar decreases were observed in dAMP activity by these mutations. However, the replacement of Thr^{39} by Ala resulted in marked *increases* in CMP and UMP activities; T39A shows CMP and UMP activities 12 and 9 times those of the wild-type enzyme, respectively. L66I and T39A/L66I having considerably decreased AMP activities show CMP and UMP activities similar to those of the wild-type enzyme. Thus the engineered adenylate kinases, especially T39A and T39A/L66I, show CMP and UMP activities/AMP activity ratio much larger than the wild-type enzyme.

Steady state kinetic parameters of the mutant and wild-type enzymes were measured for AMP, CMP, and UMP (Table II). Interestingly, the V_{max} value for AMP of L66I is about twice that of the wild-type enzyme, whereas the mutation caused an increase in the K_m value for AMP. Other kinetic parameters of this mutant enzyme are comparable to those of the wild-type enzyme. The increased k_{cat}/K_m values for CMP and UMP of T39A and T39A/L66I are attributable to both the decreased K_m values and the increased V_{max} values; a more pronounced increase in the k_{cat}/K_m value of T39A is contributed from its V_{max} value much larger than that of T39A/L66I. The V_{max} value of T39A for CMP is comparable to that of UMP-CMP kinase, although the K_m value for CMP of T39A (7.7 mM) is much larger than that of UMP-CMP kinase (0.1 mM) [4].

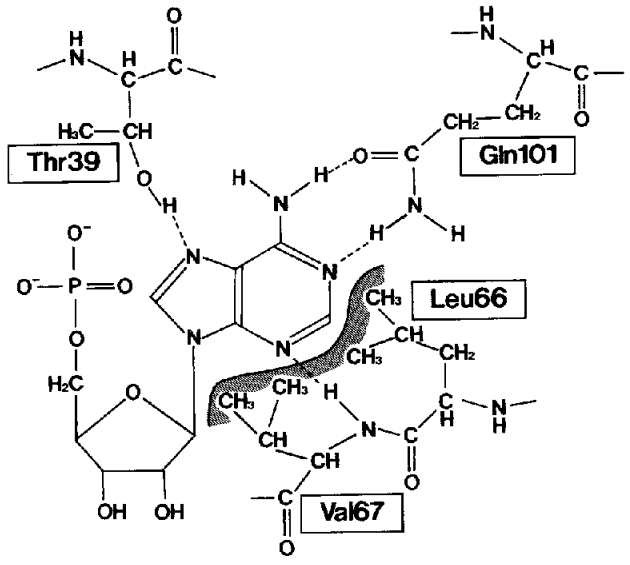

Fig. 1. Schematic drawing of the hypothetical interactions between AMP and chicken muscle adenylate kinase, constructed based on a combination of the X-ray crystallographic data [6] and the results of our present and previous site-directed mutagenesis studies [1,2]. Broken lines indicate hydrogen bonds between enzyme and AMP, and the shaded area corresponds to an hydrophobic region contributing to hydrophobic interactions between enzyme and AMP.

Table I

Specific activities for nucleoside monophosphates[a]

Enzyme	AMP	dAMP		CMP		UMP	
	unit/mg	unit/mg	(%)	unit/mg	(%)	unit/mg	(%)
Wild	1900	170	(8.9)	5.7	(0.30)	0.15	(0.008)
T39A	1600	32	(2.0)	66	(4.1)	1.3	(0.081)
L66I	720	2.0	(0.28)	3.4	(0.47)	0.021	(0.003)
T39A/L66I	120	0.27	(0.23)	19	(16)	0.16	(0.13)

[a] Specific activities for dAMP, CMP, and UMP relative to that for AMP of each enzyme are shown in parentheses.

Table II

Kinetic parameters for AMP, CMP, and UMP[a]

Enzyme	AMP			CMP			UMP		
	K_m (mM)	V_{max} (unit/mg)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	K_m (mM)	V_{max} (unit/mg)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	K_m (mM)	V_{max} (unit/mg)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
Wild	0.17	2000	4300	21	100	1.7	33	5.0	0.053
T39A	0.30	2200	2700	7.7	640	30	35	49	0.50
L66I	3.0	3500	420	35	150	1.5	78	1.6	0.007
T39A/L66I	5.0	810	58	8.8	210	8.6	11	3.4	0.11

[a] Apparent values obtained with Mg-ATP at a fixed concentration of 2 mM.

4. DISCUSSION

Diederichs and Schulz [6] suggested based on X-ray crystallographic data that the side-chain O atom of Ser^{36} in the bovine inner mitochondrial adenylate kinase AK3 (corresponding to Thr^{39} in the chicken muscle enzyme) is located within a hydrogen bond-forming distance to the purine ring N7 atom of bound AMP. Yan et al. [9] also reported that Thr^{39} might be located at the position close to the adenine ring of bound AMP but without forming a hydrogen bond. We have suggested in the site-directed mutagenesis studies that both Leu^{66} and Val^{67} forming a 'hydrophobic cap' in the chicken muscle enzyme interact with the adenine ring of AMP and assist in determining its exact location [1,2], as in Tyr^{42} and Tyr^{45} of RNase T1 that play a role of shielding the guanine ring from solvent [10].

Fig. 1 schematically illustrates the hypothetical model for the interactions between chicken muscle adenylate kinase and AMP, as constructed based on a combination of the X-ray crystallographic data [6] and the results of our present and previous mutagenesis studies [1,2]. Thr^{39} locates close to the adenine ring of AMP but a hydrogen bond between them may not be necessary for AMP binding. Rather, it is tempting to speculate on a role for Thr^{39} of hindering a pyrimidine in binding to the AMP-binding site; therefore, the mutation of this residue to Ala with a smaller side chain facilitates the accommodation of a pyrimidine at this site.

Our previous finding that the replacement of Gln^{101} by other amino acids increased the specific activity for UMP [2] led to try the triple mutant enzyme by a combination of the replacements of Thr^{39} by Ala, Leu^{66} by Ile, and Gln^{101} by Asn. However, the purified enzyme showed a perturbed CD spectrum in a region from 200 to 320 nm, and had very low activities for all nucleoside monophosphates.

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