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Mechanism of Adenylate Kinase

THE CONSERVED ASPARTATES 140 AND 141 ARE IMPORTANT FOR TRANSITION STATE STABILIZATION INSTEAD OF SUBSTRATE-INDUCED CONFORMATIONAL CHANGES*

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The goal of this work is to evaluate the quantitative contribution of Asp-140 and Asp-141 to conformational changes and/or substrate stabilization in the catalysis by chicken muscle adenylate kinase (AK), by use of kinetic and structural analysis of the single alanine mutants D140A and D141A as well as the salt bridge double mutants R138M,D140A and R132M,D141A. The single mutants D140A and D141A displayed small increases in K_m (6–12-fold) and moderate decreases in k_{cat} (17–78-fold). Kinetic analysis with the slowly reacting substrate system MgATP and dAMP suggested that the decrease in k_{cat} is not caused by a decrease in the conformational step(s) relative to the chemical step. Most of the kinetic constants of the double mutants R138M,D140A and R132M,D141A are similar to those of the single mutants R138M and R132M, respectively. Detailed analysis by two-dimensional NMR indicated no appreciable changes in the conformations of the free enzyme or the complex with $MgAP_5A$ (where AP_5A is P^1,P^5-bis(5′-adenosyl)pentaphosphate), for both single and double mutants. These results taken together suggest that Asp-140 and Asp-141 are unimportant for substrate-induced conformational changes in AK; their roles are mainly to assist Arg-138 and Arg-132 in stabilizing the transition state. The structural results also suggest that AK is a flexible enzyme.

As many of the active site residues directly involved in stabilizing the substrates have been identified for adenylate kinase (AK)[1, 2] (Tsai and Yan, 1991; Müller and Schulz, 1992; Shi *et al.*, 1993), we have shifted our emphasis of the structure-function analysis of AK to residues whose functions are less obvious. The residues targeted for this work are Asp-140 and Asp-141 of chicken muscle AK (AK1). Although these 2 residues are absolutely conserved among the known sequences of AK (Schulz, 1987), their side chains point away from the substrate binding cleft in the crystal structure of the *free* AK1, as shown in Fig. 1. The potential importance of these 2 residues was noted when the crystal structure of *Escherichia coli* AK (AKe) complexed to AP_5A was solved at 1.9 Å (Müller and Schulz, 1992). In this crystal structure Asp-158 and Asp-159 (corresponding to Asp-140 and Asp-141, respectively, in AK1) form salt bridges with Arg-156 and Arg-123, respectively, as shown in Fig. 2. The 2 arginine residues (positions 156 and 123) interacting with the aspartates in AKe correspond to Arg-138 and Arg-132, respectively, in AK1. Site-directed mutagenesis studies of AK1 have established that these 2 arginine residues are critically important in stabilizing the transition state (Yan *et al.*, 1990a; Dahnke *et al.*, 1992). AK1 differs from AKe and AKy in lacking a 30-residue loop inserted near residue 132 (Schulz, 1987; Haase *et al.*, 1989), and the crystal structure of AK1·$MgAP_5A$ is not available. However, the similarity between AK1 and AKe in all other aspects of structure and function suggests that the induced-fit process should also bring Asp-140 and Asp-141 to form salt bridges with Arg-138 and Arg-132, respectively, in the AK1·$MgAP_5A$ complex, in analogy to AKe·$MgAP_5A$.

The objective of this work is to use site-directed mutagenesis to examine the quantitative contributions of Asp-140 and Asp-141 to the structure and function of AK1. The possible roles of the two aspartate residues are (i) to help orient the arginines for stabilizing the transition state and (ii) to participate in substrate-induced conformational changes. The latter was particularly emphasized in the structural study of AKe by Müller and Schulz (1992). We are also particularly interested in the conformational aspect, since so far we have been unable to definitively disrupt the induced-fit process of AK by a point mutation. This property of AK is in contrast to that of phospholipase A_2, whose conformation is easily perturbed by point mutations (Dupureur *et al.*, 1992a; Li and Tsai, 1993). The results, however, indicate that neither aspartate is important for conformational changes. Instead, the main function of both residues are to assist the two arginines in stabilizing the transition state during catalysis. These results are discussed in relation to the mechanism of catalysis, as well as the conformational flexibility of AK.

EXPERIMENTAL PROCEDURES

Materials and Construction of Mutants—The *E. coli* expression system for AK was provided by Dr. Nakazawa (Tanizawa *et al.*, 1987). The oligonucleotides required for construction of mutants were obtained from Bio-Synthesis, Inc. and used without further purification. The sequences of the oligonucleotides for D140A and D141A are GGCGGGTGGCAGACAACGAGG and GGGTGGACGCCAACGAGGA, respectively. The double mutants R132M,D141A and R138M,D140A were constructed using the single mutants D141A and D140A, respectively, as a template with the oligonucleotides CTGCTGAAGATGGGAGAGACC

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[1] The abbreviations used are: AK, adenylate kinase; AP_5A, P^1,P^5-bis(5′-adenosyl)pentaphosphate; 1D, one-dimensional; 2D, two-dimensional; COSY, correlated spectroscopy; Gdn-HCl, guanidine hydrochloride; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser-enhanced spectroscopy; WT, wild-type.

[2] The AK from different sources are abbreviated as follows: from muscle, AK1; from *E. coli*, AKe; from yeast, AKy. Although chicken muscle AK has one additional residue near the N-terminal (Kishi *et al.*, 1986), the Met-1 residue is absent in the chicken muscle AK expressed in *E. coli* (Tanizawa *et al.*, 1987). This makes numbering of chicken muscle AK consistent with other AK1.

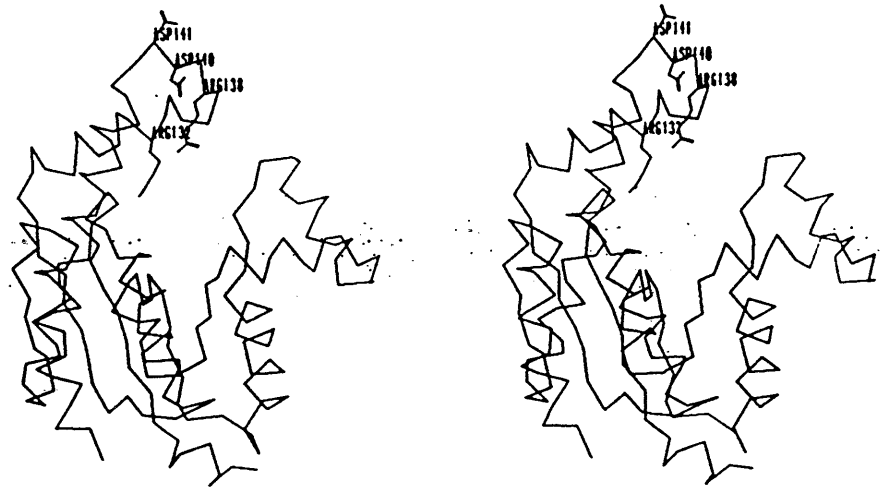

FIG. 1. **Stereoview of the backbone of the 2.1 Å structure of crystal form A of free AK1 for porcine muscle (Dreusicke *et al.*, 1988) with side chains of Arg-132, Arg-138, Asp-140, and Asp-141.**

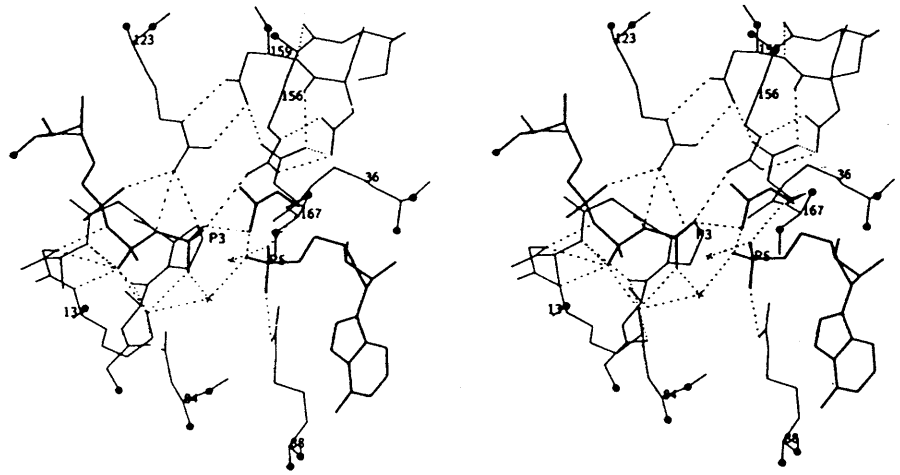

FIG. 2. **Stereoview of the partial structure of the AKe·AP_5A complex illustrating the presence of salt bridge interactions Asp-158...Arg-156 and Asp-159...Arg-123.** The residues 123, 156, 158, and 159 in AKe correspond to 132, 138, 140, and 141, respectively, in AK1. Reproduced with permission from Müller and Schulz (1992).

TABLE I

Summary of steady-state kinetic data for D140A, D141A, R138M,D140A, and R132M,D141A

Numbers in parentheses indicate the ratios between the mutant and WT. Only the large changes are indicated.

	k_{cat}[a]	$K_{(MgATP)}$[a]	$K_{(AMP)}$[a]	$K_{i(MgATP)}$[a]	$K_{i(AMP)}$[a]	$k_{cat}/K_{(MgATP)}$	$k_{cat}/K_{(AMP)}$
	s^{-1}	mM	mM	mM	mM	$s^{-1}\ M^{-1}$	$s^{-1}\ M^{-1}$
WT[b]	650	0.042	0.098	0.16	0.37	15.5×10^6	6.6×10^6
D140A	8.3 (0.013)	0.25 (5.9)	0.55 (5.6)	0.17	0.15	3.3×10^4 (2.1×10^{-3})	1.5×10^4 (2.3×10^{-3})
D141A	38 (0.086)	0.51 (12)	0.84 (8.6)	0.15	0.24	7.4×10^4 (4.7×10^{-3})	4.5×10^4 (6.8×10^{-3})
R138M,D140A	0.04 (6.1×10^{-5})	0.30 (7.1)	1.4 (14)	0.17	0.74	133 (8.6×10^{-6})	28 (4.3×10^{-6})
R132M,D141A	0.04 (6.1×10^{-5})	1.2 (28)	1.8 (18)	0.70	1.0	34 (2.2×10^{-6})	23 (3.5×10^{-6})
R138M[c]	0.05 (0.9×10^{-4})	0.40 (9.5)	2.2 (23)	0.085	0.47	120 (7.7×10^{-6})	22 (3.3×10^{-6})
R132M[d]	0.08 (1.3×10^{-4})	0.056	0.053	0.18	0.17	1.5×10^3 (1.0×10^{-4})	1.6×10^3 (2.4×10^{-4})

[a] The error limits are estimated to be 5% for k_{cat} and ±10% for K and K_i.
[b] From Tian *et al.* (1990).
[c] From Yan *et al.* (1990a).
[d] From Dahnke *et al.* (1992).

and GACCAGCGGGATGGTGGCAGAC, respectively. The construction of these mutants was identical to methods used previously (Tian *et al.*, 1988).

Purification of Enzymes and Steady-state Kinetics—D140A, D141A, and R138M,D140A were purified essentially as described by Tian *et al.* (1988). The purification of R132M,D141A was identical to WT in all aspects except that the pH of the phosphocellulose P-11 column was changed to 7.4. The enzymes were assayed by monitoring formation of ADP with pyruvate kinase/lactic dehydrogenase as the coupling system (Rhoads and Lowenstein, 1968) and the experimental details have been described previously (Tian *et al.*, 1988). The kinetic parameters were obtained by varying the concentrations of both substrates and the data analyzed according to the equation of Cleland (1986). The K and K_i values (Michaelis and dissociation constants, respectively) obtained from this analysis are close to the K_m (obtained by saturating one substrate) and K_d values (determined by titration studies with NMR), respectively for WT AK (Sanders *et al.*, 1989; Tian *et al.*, 1990).

Proton NMR Methods—All NMR experiments were performed at 27 °C on a Bruker AM-500 spectrometer. Chemical shifts were referenced to internal sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4. Sample preparation and titration procedures are identical to methods described previously (Yan *et al.*, 1990b), except for the change to pH 7.5 for 2D experiments. For 1D experiments, the spectral width was 10.5 ppm, and 16,000 data points were recorded in the time domain. Two-dimensional NMR experiments were conducted using standard pulse sequences and phase cycling, as described for NOESY (Bodenhausen *et al.*, 1984) and COSY (Marion and Wüthrich, 1983). All 2D spectra were obtained in the phase-sensitive mode and a 2048 × 1024 data matrix was recorded for each experiment and zero-filled to 4096 × 2048. The mixing time was 200 ms for NOESY experiments. Data processing for 2D spectra was carried out with FELIX (Hare Research) on an Iris Silicon Graphics work station.

Gdn-HCl-induced Denaturation—The reversible denaturation of mutants with the chemical denaturant Gdn-HCl was monitored by follow-

ing changes in ellipticity at 222 nm of CD spectra obtained with a JASCO J-500C polarimeter. The sample conditions and experimental procedures are identical to those described previously (Dahnke *et al.*, 1992). The conformational stabilities were determined according to Pace (1986).

RESULTS AND DISCUSSION

Rationale for Detecting Changes in the Induced Fit Process

Although the criteria for determining enzyme-substrate interactions have been well established, the experimental methods to assess perturbations in the conformational changes of a mutant enzyme remain to be defined. In our view, the most affirmative approach would be structural analysis of the free enzyme, binary complexes (AK + AMP and AK + MgATP) and ternary complex (AK + $MgAP_5A$). A decisive perturbation in the induced fit mechanism would require that the conformation of free mutant enzyme be unperturbed relative to WT and that structural differences arise starting at either the binary or the ternary complex. An intrinsic difficulty in this approach is that the perturbation in conformation is a qualitative and relative term, even if tertiary structures can be solved for each of the complexes. However, NMR is known to be an overly sensitive technique in detecting changes in protein conformations. A global conformational change would exhibit wide spread shifts in both aromatic-aromatic and aromatic-aliphatic cross-peaks, whereas a localized change may only effect a small number of resonances. In our conservative judgment, we consider that the conformation of a mutant is practically "unperturbed" globally if its NOESY spectrum resembles that of WT and if <5% of the assigned resonances shift by >0.1 ppm relative to WT.

The second criterion which could be used to assess kinetic differences in conformational change is the rate of substrate binding to the free or binary forms of the enzyme. Ideally, these pre-steady-state kinetic constants are best obtained by rapid mixing stopped-flow kinetic techniques, but a simpler and qualitative method to address this issue is to determine if the rate-limiting step(s) change significantly between WT and the mutant enzyme. Kinetic analysis with the substrate system ATP and dAMP or dATP and AMP has been used in the assessment of the rate-limiting steps of WT AK (Tian *et al.*, 1990). These slowly reacting analogues are more likely to increase the energy barrier of the chemical conversion than that of the conformation change. The ratio of catalytic rates (k_{cat}/K_m) between the substrate systems AMP and dAMP is approximately 80-fold for WT. If a mutation changes the kinetic mechanism so that the conformational change step(s) are fully rate limiting for natural substrates, then the differences in k_{cat}/K_m between AMP and dAMP will decrease from 80 and approach 1 if the conformational step is still fully rate limiting in the presence of dAMP.

The third criterion is that a perturbation in the induced fit mechanism could lead to increases of K_m for *both* substrates. The best example of a mutant in this category is R149M (K_m increases by 130-fold for both substrates) (Yan *et al.*, 1990b). However, we believe that symmetrical increases in K_m are *insufficient* evidence for a perturbation in the induced fit mechanism. It could also be caused by a disruption in a localized enzyme-substrate interaction, as long as this interaction (in WT) does not come in place until the *ternary* complex is formed. Thus, we have not used this criterion to suggest that Arg-149 is

TABLE II
Summary of the saturation kinetic data for WT, D140A, and D141A using the substrate system MgATP and dAMP

	Substrates	k_{cat}[a]	K_m[a, b]	k_{cat}/K_m	k_{cat}/K_m (ratio), AMP:dAMP
		s^{-1}	mM	$s^{-1}\ M^{-1}$	
WT	MgATP + AMP	500	0.15	3.3×10^6	80:1
WT	MgATP + dAMP	87	2.1	4.1×10^4	
D140A	MgATP + AMP	6	1.4	4.3×10^3	170:1
D140A	MgATP + dAMP	0.10	3.9	25	
D141A	MgATP + AMP	22	1.8	1.2×10^4	460:1
D141A	MgATP + dAMP	0.27	10.3	26	

[a] The error limits are estimated to be 5% for k_{cat} and ±10% for K_m.
[b] This K_m corresponds to the apparent Michaelis constant for AMP or dAMP.

TABLE III
Free energies of unfolding induced by guanidine hydrochloride

The data were analyzed according to the equation $\Delta G_d = \Delta G_d^{H_2O} - m$[Gdn-HCl] (Pace, 1986), where ΔG_d is the Gibbs free energy of denaturation at various concentrations of Gdn-HCl, $\Delta G_d^{H_2O}$ is that extrapolated to zero concentration of Gdn-HCl, and m is a constant. $D_{1/2}$ is the concentration of Gdn-HCl at the midpoint of unfolding. The error limit for $\Delta G_d^{H_2O}$ is estimated to be ±0.3 kcal/mol.

	$\Delta G_d^{H_2O}$	$D_{1/2}$	m
	kcal/mol	M	kcal/mol·M
WT	4.5	0.81	5.7
D140A	5.2	0.83	6.3
D141A	5.4	0.80	6.6
R138M,D140A	5.6	0.83	6.7
R132M,D141A	5.7	0.84	6.9

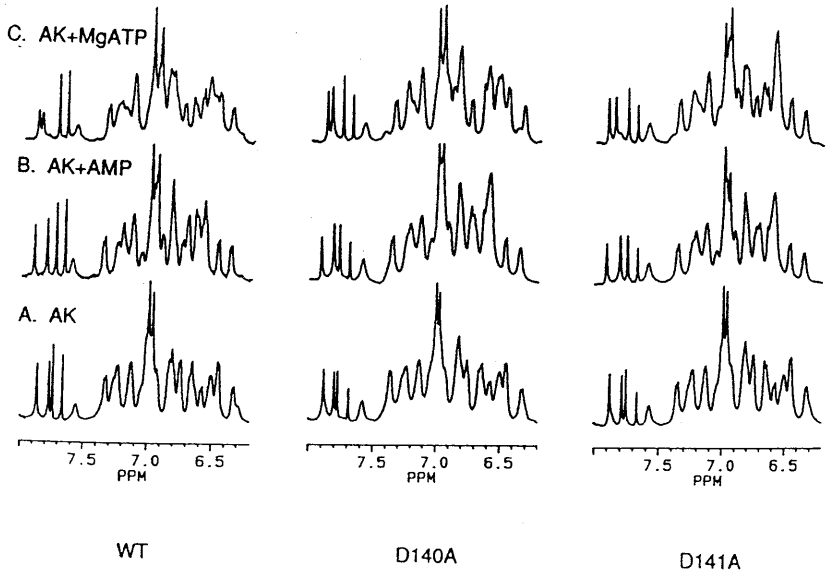

FIG. 3. **One-dimensional proton NMR spectra of the aromatic protons of WT (*left*), D140A (*middle*), and D141A (*right*) as free (*A*), AMP-complexed (*B*), and MgATP-complexed (*C*).** The spectra of WT are reproduced from Sanders *et al.* (1989). Spectra were obtained at pH 7.8, 27 °C, and the free induction decays were processed with a 1 Hz line broadening. The concentrations for AK/AMP (in mM) are 1.3/3.4 D140A and 1.4/3.4 D141A and for AK/ATP/Mg^{2+} (in mM) are 1.4/5.5/11 D140A and 1.4/5.4/11 D141A.

TABLE IV
Binding constants obtained from proton NMR substrate titration

	K_d(MgATP)[a]	K_d(AMP)[a]
WT[b]	0.17	0.50
D140A		0.49
D141A	0.18	0.39

[a] The error limit for K_d is estimated to be ±20%.
[b] From Sanders *et al.* (1989).

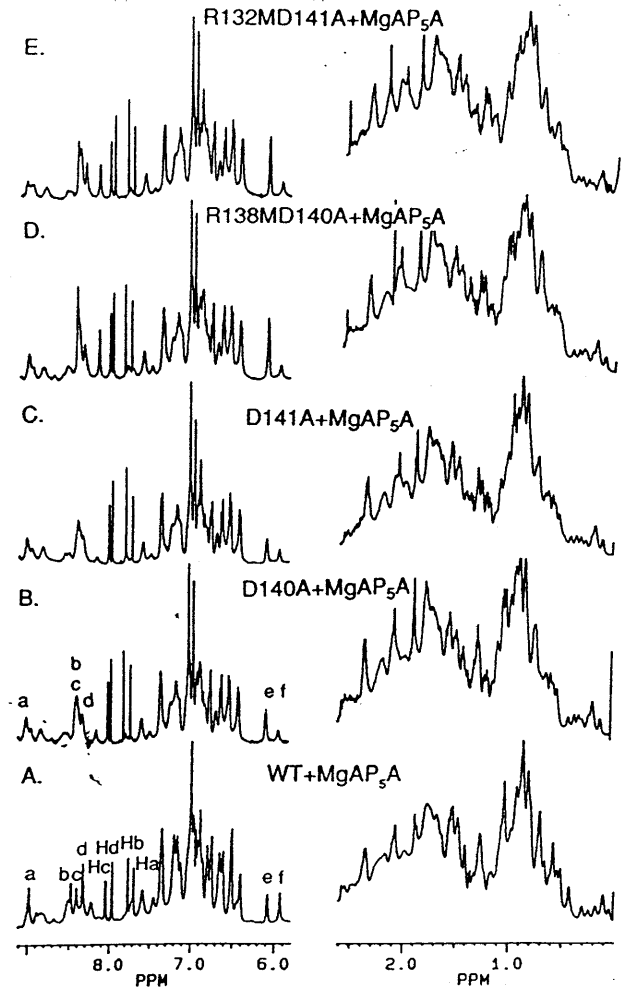

FIG. 4. **One-dimensional proton NMR spectra of the $MgAP_5A$ complexes of WT (*A*), D140A (*B*), D141A (*C*), R138M,D140A (*D*), and R132M,D141A (*E*).** The spectrum of WT is reproduced from Yan *et al.* (1990b). The concentrations for AK/AP_5A/Mg^{2+} (in mM) are 2.0/3.5/5.9 (*B*), 2.1/3.4/5.9 (*C*), 2.4/4.4/6.1 (*D*), and 2.2/4.0/6.2 (*E*). *Peaks a–f* are due to bound $MgAP_5A$: H_2(I) (*peak a*); H_8(I) (*peak b*); H_8(II) (*peak c*); H_2(II) (*peak d*); H_1'(II) (*peak e*); H_1'(I) (*peak f*). Intermediate exchange rates for all spectra except WT cause signal broadening and overlap of *peaks b* and *c*. It should be noted that the H_8(free) and H_1'(free) resonances overlap H_8(II) and H_1'(II) resonances, respectively, whereas H_2(free) resonates at 8.19 ppm. Spectra were obtained at pH 7.5, 27 °C, and the free induction decays were processed with Gaussian multiplication (*LB* = −5, *GB* = 0.1).

involved in conformational change, even though it would be *consistent with* the increases in K_m. Since the k_{cat} of R149M also decreases dramatically, we believe the role of Arg-149 is mainly to stabilize one or both of the substrates, *starting at the ternary complex.*

Kinetic Analysis of Mutants

The steady-state kinetic properties of D140A and D141A are shown in Table I. Replacement of Asp-140 or Asp-141 with an alanine produces moderate decreases in k_{cat} (78- and 17-fold, respectively) and small to moderate increases (6–12-fold) in K_m for both substrates. The dissociation constants remain unperturbed for AMP and MgATP in both mutants.

As described in the previous section, the increase in K_m for both substrates could be caused by either a perturbation in the induced fit mechanism or a disruption in enzyme-substrate interactions at the ternary complex. In either case, the modest changes in K_m would suggest that the roles played by either aspartate residue are quite small. On the other hand, the decreases in k_{cat} (particularly that of D140A) are more significant. Without the current structural knowledge regarding the interactions between AK and its substrates at the active site, the kinetic data of these two mutants could have led one to conclude (after demonstrating lack of global conformational changes in a later section) that the 2 aspartate residues are involved in catalysis by interacting *directly* with substrates at the transition state. Since this is highly unlikely on the basis of the current knowledge, the decreases in k_{cat} could be caused by one or more of the following three factors: (*a*) the conformational changes becomes the rate-limiting step, (*b*) a global conformational change in the free form of the mutants or in their

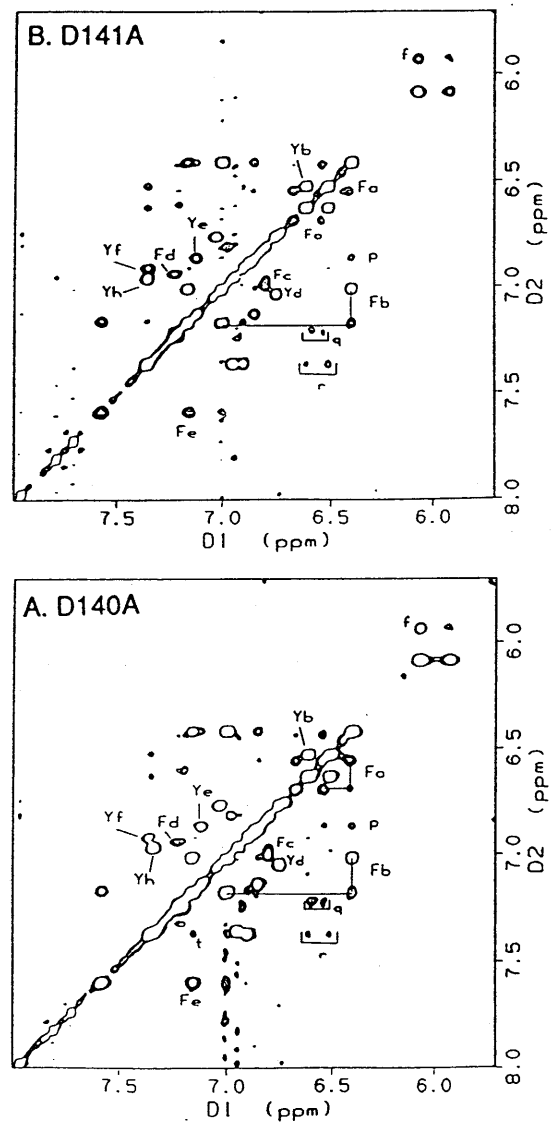

FIG. 5. **NOESY spectrum of the complex of D140A (*A*) and D141A (*B*) with $MgAP_5A$ (same samples as used for Fig. 4).** The *cross-peak f* arises from exchange between the two H_1' protons of bound $MgAP_5A$, whereas *cross-peaks p–t* arise from interresidue NOEs, as described previously (Yan *et al.*, 1990a, 1990b). It should be noted that *cross-peaks s* and part of *q* are not resolved in *A* and *cross-peaks s, t,* and part of *q* are not resolved in *B*.

TABLE V

Chemical shifts of the aromatic residues of the complexes of D140A·MgAP$_5$A and D141A·MgAP$_5$A (pH 7.5)

The values underlined are the resonances which differ by >0.02 ppm from the corresponding resonances of WT·MgAP$_5$A at pH 7.5, and the magnitudes of the differences are shown in parentheses.

Spin system	Assignment[a]	D140A·MgAP$_5$A			D141A·MgAP$_5$A		
Fa	12	6.42	6.55	6.69	6.42	6.55	6.69
Fb	163	6.41	7.01	7.17	6.40 (−0.03)	7.00	7.17
Fc	90	6.81	6.95	7.00	6.81	6.95	6.99
Fd	105	6.94		7.23	6.94		7.23
Fe	183		7.15	7.59	7.00	7.15	7.59
Yb	34	6.52	6.63		6.52	6.63	
Yd	189	6.75	7.04		6.75	7.04	
Ye	164	6.86	7.13		6.86	7.13	
Yf	32	6.91	7.37		6.90	7.36	
Yh	154	6.95	7.35		6.95	7.37 (+0.03)	
Ha	30	7.01 (+0.03)	7.72 (+0.08)		7.01 (+0.03)	7.71 (+0.07)	
Hb	7	6.95 (+0.06)	7.81 (+0.12)		6.94 (+0.05)	7.79 (+0.10)	
Hc	36		8.00		6.83	8.00	
Hd	8	6.74	7.97 (+0.03)		6.74	7.96	

[a] Assignment is based upon WT·MgAP$_5$A at pH 7.1 (Byeon *et al.*, 1993); due to the difference in pH, the histidine assignments are tentative.

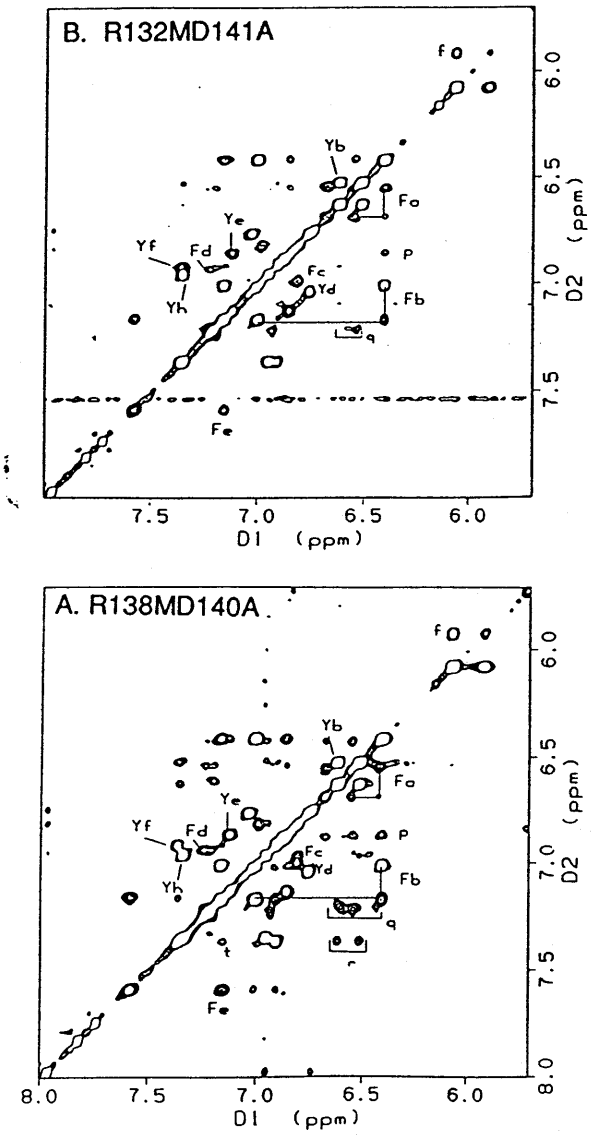

FIG. 6. **NOESY spectrum of the complex of R138M,D140A (*A*) and R132M,D141A (*B*) with MgAP$_5$A (same samples as used for Fig. 4).** It should be noted that *cross-peak s* is not resolved in *A*, and *cross-peaks r, s, t,* and part of *q* are not visible in *B*.

complexes with substrates, and (*c*) Asp-140 and Asp-141 interact *indirectly* with substrates at the transition state. These three possibilities are probed in the following three sections.

Conformational Change Does Not become Rate-limiting in Mutants

A summary of the results of saturation kinetics with the substrate system MgATP and dAMP is shown in Table II. The ratio of rates (k_{cat}/K_m) between AMP and dAMP is compared for WT, D140A, and D141A in the last column. Since these ratios are larger for both D140A and D141A, this result suggests that the chemical step becomes more rate-limiting for these two mutants and argues against possibility *a*.

There Is No Global Conformational Change in the Mutants

The Free Enzyme—The 1D proton NMR spectra of the aromatic resonances of the free D140A and D141A are compared with WT in Fig. 3*A*. The spectra of the free enzymes are quite similar to WT, suggesting no change in the global conformation of these mutants. The free energy of unfolding was determined for both single and double mutants by monitoring the change in ellipticity of CD spectra obtained with varying concentrations of Gdn-HCl. The data, summarized in Table III, are similar to the WT values and suggest that these aspartates are unimportant for the conformational stability of AK.

Binary Complexes—The spectra of the AMP complexes (Fig. 3*B*) exhibit only slight differences in the range 6.5–6.8 ppm. The MgATP complexes (Fig. 3*C*) show greater perturbation than the AMP complex, with notable differences in the range of 6.4–6.8 ppm for D140A and 6.3–6.8 ppm for D141A.

The dissociation constants determined from NMR substrate titration are shown in Table IV. Dissociation constants for AMP were comparable with the K_i determined from kinetic analysis for both mutants. The titrations of D140A and D141A with MgATP exhibit a very different pattern than that characteristically associated with the WT titration with MgATP (Sanders *et al.*, 1989), and fitting of the data to estimate K_d for D140A was unsuccessful.

In summary, there appear to be some structural perturbations in the binary complexes of both mutants with MgATP. The fact that the conformation of a mutant is nearly identical to that of WT but displays small deviation in substrate complexes has been observed previously for many of our AK mutants. This phenomenon may fit into the first criterion described under "Rationale." However, the question is whether the deviation is small and localized or large and global. It is difficult to definitively address this question on the basis of 1D spectra. Two-dimensional NOESY spectra of the MgATP complexes were not obtained, since MgATP tends to hydrolyze during prolonged experiments. Our approach in the past has been to perform

TABLE VI

Chemical shifts of the aromatic residues of the complexes of R138M,D140A·MgAP$_5$A and R132M,D141A·MgAP$_5$A (pH 7.5)

The values underlined are the resonances which differ by >0.02 ppm from the corresponding resonances of WT·MgAP$_5$A at pH 7.5, and the magnitudes of the differences are shown in parentheses.

Spin system	Assignment[a]	R138M,D140A·MgAP$_5$A			R132M,D141A·MgAP$_5$A		
Fa	12	6.42	6.55	6.68	6.41	6.55	6.68
Fb	163	6.41	7.01	7.17	6.41	7.01	7.17
Fc	90	6.81	6.96	7.00	6.82	6.96	7.00
Fd	105	6.94		7.24	6.94		7.23
Fe	183	7.01	7.15	7.59	7.00	7.16	7.59
Yb	34	6.52	6.63		6.51	6.62	
Yd	189	6.75	7.04		6.75	7.04	
Ye	164	6.86	7.13		6.85	7.13	
Yf	32	6.91	7.37		6.91	7.36	
Yh	154	6.95	7.35		6.95	7.36	
Ha	30	7.01 (+0.03)	7.73 (+0.09)		7.00	7.71 (+0.07)	
Hb	7	6.95 (+0.06)	7.82 (+0.13)		6.94 (+0.05)	7.79 (+0.10)	
Hc	36	6.84	8.00		6.84	8.01	
Hd	8	6.75	7.96		6.74	7.96	

[a] Assignment is based upon WT·MgAP$_5$A at pH 7.1 (Byeon *et al.*, 1993); due to the difference in pH, the histidine assignments are tentative.

detailed analysis on the ternary complex E·MgAP$_5$A, which is described in the following section.

Ternary Complexes—The MgAP$_5$A complexes with D140A and D141A were analyzed by 1D proton NMR and are compared to the WT enzyme in Fig. 4, *A–C*. Some differences in the 1D spectra of these complexes are observed from 6.4–6.8 ppm. Fig. 5 illustrates the partial NOESY spectra of D140A·MgAP$_5$A (*A*) and D141A·MgAP$_5$A (*B*); the aromatic spin systems identified from these spectra are found in Table V. Based upon the comparison with the corresponding NOESY spectrum and chemical shifts of WTAK·MgAP$_5$A (Yan *et al.*, 1990a, 1990b), it can be safely concluded that there are no apparent differences in structure between D140A and WT in the ternary complex. The only significant variances are found with the histidines, which commonly differ in chemical shift from WT despite careful attempts to prepare all NMR samples at the same pH and ionic strength. The spectrum of D141A is also very similar to WT in the ternary complex, with only two small differences (0.03 ppm) in spin systems Fb and Yh.

The above results taken together suggest that the conformations of the mutants have not been perturbed in the free enzyme and that noticeable but insignificant perturbations occur in substrate complexes. In a comparative sense, the perturbations in the NMR spectra of D140A and D141A and their complexes with substrates are no more extensive than most of our previous AK mutants which have been concluded as "no global perturbation in conformations" and used as a basis for quantitative interpretation of the kinetic data. The results of structural analysis therefore argue against possibility *b*, *i.e.* the decrease in k_{cat} should not be caused by global conformational changes or perturbations in the induced fit mechanism.

Asp-140 and -141 Stabilize the Transition State, but Indirectly

Although possibility c can be concluded by the process of elimination, it was further tested by constructing double mutants R138M,D140A and R132M,D141A. The residues Arg-138 and Arg-132 are known to contribute significantly to the stabilization of the transition state. If the roles of Asp-140 and Asp-141 are mainly to stabilize the transition state by *indirectly* interacting with Arg-138 and Arg-132, respectively, the double mutants should display similar kinetic parameters to the corresponding single mutants R138M and R132M. On the other hand, if Asp-140 and Asp-141 play independent roles in transition-state stabilization or in conformational changes, the effects on the double mutants should be additive or worse. The kinetic parameters of the salt bridge double mutations along with those of the single arginine mutations R138M and R132M are also shown in Table I. In the case of R138M,D140A, all of the kinetic constants agree with that of R138M within a factor of 2. In the case of R132M,D141A, k_{cat} also agrees with the single mutant, whereas K_m increases in the double mutant (18- and 28-fold for AMP and MgATP, respectively), but not the single mutant R132M. Thus, with the exception of the K_m of D141A, the aspartate residues play no additional roles than assisting the corresponding arginine residues.

The conformations of the double mutants R138M,D140A and R132M,D141A were also examined with MgAP$_5$A. The 1D spectra are shown in Fig. 4, *D* and *E*, and the 2D NOESY spectra are shown in Fig. 6. Careful analysis reveals that the spectra of these double mutants are similar to the corresponding single mutants D140A and D141A in the ternary complex. The partial assignments of intra- and interresidue cross-peaks were made from COSY (not shown) and NOESY spectra, and the chemical shifts of aromatic spin systems are listed in Table VI. Examination of the data in Table VI reveals that neither of the double mutations are structurally perturbed. The results again argue against significant conformational roles of the aspartate residues.

Conformational Change: What Can Be Learned from Mutagenesis?

Although we have enjoyed considerable success in removing most of the catalytic activity with carefully chosen single mutations, this same goal with respect to conformational change has not been realized for these aspartate mutants. It is particularly intriguing that even the double mutants do not display perturbed conformations. However, these results do not necessarily imply that Asp-140, Asp-141, Arg-132, and Arg-138 are not involved in conformational changes. Conformational change is an integrated process involving a large number of residues. Accordingly, the process may be difficult to be disrupted by mutation of 1 or 2 residues.

Adenylate Kinase Is Unstable but Flexible

Of the >20 mutants we have characterized by NMR, including those described in this paper, only one (K21M, found in the P-loop) has displayed significant conformational changes in the free enzyme (Tian *et al.*, 1990). This brings up another issue; if the conformation of AK is so tolerant of single or double mutations, should we be less concerned with the potential global changes in site-directed mutagenesis studies in general? Our view is that AK is an unstable but flexible enzyme. The free energy of denaturation, which lies between 5–15 kcal/mol for

most enzymes (Pace, 1990), is only 4.5 kcal/mol for AK1. AK1 is also known to be thermally very unstable in our experience. However, AK has no disulfide bonds and undergoes large conformational changes during catalysis. It is likely that the flexible nature of the conformation as required for catalysis is responsible for its relative instability and its tolerance to mutagenesis.

As a sharp and interesting contrast, phospholipase A_2 from bovine pancreas (14 kDa) possesses seven disulfide bonds. Analyses of crystal structures of the free form and the inhibitor-bound form of the enzyme suggested no major conformational change during catalysis (Scott *et al.*, 1990). Thus phospholipase A_2 appears to be rigid or inflexible in conformation. On the basis of the same argument for AK, this inflexible enzyme could be stable but intolerant of mutagenesis. Both properties have been observed. The enzyme is thermally stable, since its purification involves heating at 70 °C for 3 min (Dutilth *et al.*, 1975); it also has a large free energy of unfolding (Dupureur *et al.*, 1992b). Among the ~45 site-specific mutants we have characterized, at least 10 have displayed significant and global conformational changes (Dupureur *et al.*, 1992a; Li and Tsai, 1993; data not shown). Thus, it can be said that *phospholipase A_2 is stable but fragile*, which is in direct contrast to the *unstable but flexible property of AK.*

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