Structural and Energetic Factors of the Increased Thermal Stability in a Genetically Engineered *Escherichia coli* Adenylate Kinase*

(Received for publication, March 27, 1998, and in revised form, May 15, 1998)

Simona Burlacu-Miron, Véronique Perrier‡, Anne-Marie Gilles‡, Elisabeth Pistotnik‡, and Constantin T. Craescu‡

From INSERM U350, Institut Curie-Recherche, 91405 Orsay Cedex, France and ‡Laboratoire de Chimie Structurale des Macromolécules (URA D1129), Institut Pasteur, 7524 Paris Cedex 15, France

Several variants of *Escherichia coli* adenylate kinase, designed to bind a Zn$^{2+}$ ion, were produced by site-directed mutagenesis. The metal binding and enzymatic properties of the engineered variants have been described (Perrier, V., Burlacu-Miron, S., Bourgeois, S., Surewicz, W. K., and Gilles, A.-M. (1998) *J. Biol. Chem.* 273, 19097–19101). Here we report the structural properties and stability changes in a 4-Cys variant which binds a Zn$^{2+}$ ion and has an increased thermal stability. CD studies indicate a very similar secondary structure content in the wild type and the engineered variant. NMR analysis revealed that the topology of the parallel β-sheet, belonging to the protein core, and of the peripheral antiparallel β-sheet are also conserved. The small local changes observed in the neighborhood of the substitution sites reflect a more compact state of the metal-binding domain. The Zn$^{2+}$-bound quadruple mutant shows an increased thermal stability, reflected in a 9 °C increase of the mid-temperature of the first cooperative unfolding step. Binding of a bisubstrate analog P$^1$P$^2$, di(adenosine-5’)-pentaphosphate increases, by about 7 °C, the midpoint of this transition in both wild type and modified variant. The NMR data suggest that the peripheral domains involved in substrate binding unfold during the first denaturation step. Urea denaturation experiments indicate an increased resistance against chemical unfolding of the Zn$^{2+}$-binding variant. In contrast, the Gibbs free energy of unfolding (at physiologically relevant conditions) of the quadruple mutant is lower than that of the wild type.

Adenylate kinases (AKs) represent a class of small (20–26 kDa) and abundant phosphotransferases involved in the reversible transfer of the terminal phosphate group from ATP to AMP. They contribute to the maintenance of a constant level of cellular adenine nucleotides, necessary for energetic metabolism and nucleic acid synthesis. From the structural point of view (Fig. 1) AKs belong to the α/β class (a five-stranded β-sheet surrounded by several α-helices), characteristic for many nucleotide-binding proteins (1). In addition to this protein core, they contain generally two smaller domains involved in the substrate binding. One is composed by two helices (α2 + α3) and contributes to the mononucleotide binding, NMP domain closing over bound AMP (residues 30–59). The other is involved in binding of ATP and, due to its large movement, closing over the bound nucleotide, it was called LID domain. In the mitochondrial and most of the bacterial variants of AK, the LID domain has 38 residues (residues 122–159 in AK) while smaller cytosolic enzymes have only an irregular loop of 11 residues (2, 3). In the AKb (a “long” kinase) the LID domain contains a small four-stranded antiparallel β-sheet (4). X-ray structures of the enzyme in complex with several ligands revealed that this domain has significantly different positions relative to the protein core, depending on the nature and number of bound ligands. Therefore, it was inferred that complexation (particularly with ATP) induces a large scale rotation/translation movement from a remote position into a close contact with the main part of the protein (3–6). Solution studies, using resonance energy transfer measurements (7), confirmed the existence of such hinge motion of the LID domain upon binding of Ap$^2$A, AMP, and ATP. Nevertheless, the short AKs conserve their catalytic activity despite the absence of the LID domain, but seems to lose the high specificity for ATP (8).

A screening of bacterial AKs has revealed that the enzymes from Gram-positive organisms bind a Zn$^{2+}$ ion *via* coordinative bonds to four side chains (Cys and Asp) situated in the β-hairpin loops of the LID domain (9). Based on sequence comparison with AK from the Gram-positive, thermophile *Bacillus stearothermophilus*, we have designed several variants of the *Escherichia coli* enzyme involving three- or four-amino acid substitutions in the LID domains, at positions His$^{126}$, Ser$^{129}$, Asp$^{146}$, and Thr$^{149}$. In the companion study (10) we showed that some of these variants bind a Zn$^{2+}$ ion and conserve the enzymatic activity. In this study we report the results of NMR and CD experiments aimed to characterize in more detail the structural consequences of the amino acid substitutions and zinc binding. The 4-Cys variant has an increased thermal and chemical (urea) stability, but the structural stability at physiologically relevant conditions is slightly reduced.

EXPERIMENTAL PROCEDURES

All chemicals were of highest purity available. Protein design, expression, and purification were described in our companion study (10).

**Circular Dichroism**—The far-UV CD spectra were recorded on a Jasco 700 spectropolarimeter in a 0.1-cm quartz cells. The spectra were obtained as an average of five runs and were corrected for the contribution of the buffer. For the thermal denaturation experiments we...
followed the ellipticity at 222 nm as a function of temperature, programmed to rise uniformly at 60 °C/h.

Urea denaturation was monitored by the changes in ellipticity at 222 nm. Aliquots of concentrated solutions of protein were added to urea solutions (0–8.8M) and the samples were incubated until equilibrium was reached at the temperature chosen for determining the unfolding curve (20 °C). The Gibbs free energy of denaturation was estimated by Equation 1

\[
\Delta G = -RT \ln K \quad \text{(Eq. 1)}
\]

where \( K \) is the equilibrium constant of denaturation at a given reaction temperature (RT) and urea concentration. The measured free energy difference is considered (11) a linear function of the denaturant concentration

\[
\Delta G = \Delta G(H_2O) - m[\text{urea}] \quad \text{(Eq. 2)}
\]

where \( \Delta G(H_2O) \) is the extrapolated value of \( \Delta G \) at zero molar denaturant, represents the intrinsic stability of the protein in absence of urea.

NMR Methods—Samples were prepared by dissolving the lyophilized protein in potassium phosphate buffer (50 mM) in \(^2\text{H}_2\text{O}\) or in \(^2\text{H}_2\text{O}\) containing 7% 2H2O at pH 6.5. NMR spectra were obtained on a Varian Unity 500 NMR spectrometer, using standard methods for pure absorption DQF-COSY (12), NOESY (13), and TOCSY experiments with 30–60-ms spin lock times (14). Proton chemical shifts were referenced to the water signal which, in our conditions, at 308 °C. NMR analysis of a protein such as AKC4 in this type of secondary structure (16). Using several two-dimensional homonuclear NMR spectra (DQF-COSY, TOCSY, and NOESY) we assigned the majority of the resonances corresponding to the two β-sheet domains in the AK and AKC4. The quality of the two-dimensional spectra and some of the sequential NOE pathways of AKC4 are shown in Fig. 2. The chemical shift values for the assigned resonances in the proton spectra of AKC4 and AK are available upon request from the authors.

Resonance assignment enabled us to identify a large number of sequential and long range dipolar interactions between specific protons of the protein. Observation of strong sequential connectivities \( d_{\alpha N}(i, i + 1) \) indicates very short distances between the corresponding protons (CaH\(^{1}\), NH\(^{1}\)), as the case for a backbone in an extended conformation. The local geometry was further characterized by the analysis of the chemical shift values. Indeed, statistical analysis of experimental NMR data on proteins, showed that the Ca protons experience a mean upfield shift of 0.4 ppm if the residue belongs to an α-helix and almost the same downfield shift value in the β-sheet or extended structures (17). Together, the sequential connectivities and the secondary shift of α protons provided a reliable delineation of the β-strands in AK and AKC4.

In addition, analysis of long range NOEs between resonances corresponding to the backbone protons revealed the relative arrangement of the secondary structure elements. In particular, the presence of long range \( \text{d}_{\alpha\beta}(i, j) \) NOE cross-peaks is characteristic for antiparallel β-sheets. The synthesis of the present NMR data is the global topology of the polypeptide chain in the two β-sheet domains, as represented in Fig. 3 for AKC4. A number of other long range dipolar interactions involving all the protons support this topology. For instance, methyl protons of Leu\(^{153}\) give NOE connectivities with amide protons of Tyr\(^{153}\), Gly\(^{144}\), and Lys\(^{145}\) and with aromatic protons of Tyr\(^{133}\).

The secondary and tertiary structure descriptions of AKC4, based on the present experimental data, are very similar to that obtained for the wild-type enzyme and are completely compatible with the crystallographic structure of AK (4). How-
ever, a number of minor spectroscopic modifications were observed between the wild-type protein and AKC4 in the LID domain. Chemical shift changes (up to 0.45 ppm) were noted for residues adjacent to the substituted positions (Ala127, Val125, and Val148). Also, dipolar interactions between amide protons of Tyr133 and Thr154 and $d_{1,2}$ methyl protons of Leu153 were observed in AKC4 but not in AKce. This suggests that Zn$^{2+}$ binding in AKC4 brings closer the two loops containing the cysteine side chains, and the movement extends over the adjacent $\beta$-strands, so that the methyl groups of Leu153 come into NOE contact with the amide proton of Tyr133. In contrast, sequential $d_{NN}(i, i+1)$ connectivities for the amide pairs 147/148 and 148/149 and the NOE between $C_{a}$H(145) and NH(153) were observed in AKce but not in AKC4 spectra.

**Investigation of the Thermal Stability**—The heat-induced denaturation curve in Fig. 4 was obtained by recording the ellipticity at 222 nm, which is the minimum in the far-UV CD spectrum for an $\alpha$-helix. The two proteins show a similar profile including an initial slow phase followed by two cooperative transitions, separated by a clear plateau. As reflected in the relative ellipticity changes, a comparable amount of secondary structure is unfolded in each step. The $T_{m}$ (the mid-temperature of the cooperative transition) for the first transition of the wild-type protein is 52.5 °C which is in good agreement with the previous data (18, 19) and close to the calorimetric value (10). The quadruple mutant shows a significantly increased mid-temperature (61.6 °C), close to the thermal transition (63 °C) observed by microcalorimetry (10). The apoAKC4 (the four Cys mutant in absence of Zn$^{2+}$) has an intermediate thermal stability between the wild type and AKC4 (Table I). Dithiothreitol, an efficient sulfhydryl reductant, at 1–10 mM has no significant effect on the denaturation transition points.

The second transition, not observed in previous studies, is less cooperative and shows more similar $T_{m}$ values for the two proteins (72.6 and 75.6 °C for AKce and AKC4, respectively). The unfolding process is entirely reversible for AKC4 and partially reversible (80%) for the wild-type protein when the tempera-

---

**Antiparallel $\beta$-sheet**

**Parallel $\beta$-sheet**

---

**FIG. 3.** Schematic representation of the global topology of the two $\beta$-sheet domains in AKC4 as defined by the present NMR analysis. The amino acids shown are those for which at least the backbone protons were assigned. The strong sequential connectivities ($d_{NN}(i, i+1)$) within the strands and the $d_{a}(i,j)$ and $d_{aa}(i,j)$ long range connectivities between $C_{a}$ protons on opposite strands are indicated by arrows.

**FIG. 4.** Temperature unfolding of Akce and AKC4 monitored by the ellipticity at 222 nm as a function of temperature. Samples are about 10 mM in 10 mM Tris/HCl buffer, pH 7.2. The data represent wild-type AKce (continuous line) and the variant AKC4 (dotted line) in absence (A) and presence (B) of Ap5A.
Ap$_5$A is a bisubstrate analog which binds simultaneously at the ATP and AMP sites of AK$_e$, giving a more compact structure in which the LID domain is in close contact with the protein core (20). To quantify the effect of its binding on the structural stability we studied the denaturation process in the presence of an equimolar concentration of Ap$_5$A. The first transition is significantly shifted toward higher temperatures for AK$_e$, apoAKC$_4$, and AKC$_4$ by about 7 °C (Fig. 4 and Table I). In contrast, no effect was observed on the second transition. A similar thermal protection was observed for a fluorescent analog of Ap$_5$A (19).

The temperature-induced unfolding of the wild type and two mutated variants was further studied by recording one-dimensional proton NMR spectra at different temperatures. The heating up to the first equilibrium intermediate state is accompanied by continuous, small changes of some resonances corresponding to nonexchangeable protons. Some of these peaks were identified in the NMR one-dimensional spectra and were assigned to precise protons of the protein (Fig. 5). One example corresponds to the aromatic protons of Tyr$^{181}$: a second peak, slightly high field shifted and of increasing intensity is observed in all three samples. Similarly, resonances from imidazole protons of His$^{172}$ undergo a small upfield shift while the high field peak from Leu$^{213}$ methyl (chemical shift = -0.16 ppm) is unchanged. At the temperature where the first unfolding transition already took place, these resonances are probes for a folded structure as their chemical shift values are very different (from 0.36 to 0.94 ppm) from the random coil values (16). We note that Tyr$^{181}$, Leu$^{213}$, and His$^{172}$ are located in helices, α8, α9, and C-ends of α7, respectively, within the protein core (Fig. 1). Their chemical shift values should be largely dominated by the ring current of neighboring aromatic side chains (Tyr$^{182}$, Tyr$^{206}$, and Tyr$^{171}$, respectively). These observations reflect only small local conformational rearrangements of the side chains within a conserved secondary and tertiary structure. Compared with the wild type, the spectral changes in the two variants AKC$_4$ and AKHC$_3$ extend over larger temperature ranges, suggesting that the Zn$^{2+}$-binding in the LID domain influences the local fluctuations in the central core.

A large number of Cα protons, resonating between 4.7 and 6.0 ppm, conserve their low field shift characteristic for β-strand secondary structure. An example is the Cα proton of Tyr$^{181}$, a residue from the β5 strand of the parallel sheet, which is clearly identified in all one-dimensional spectra at 5.77 ppm (Fig. 5). In the case of AKC$_4$, this observation is indicative of persistence of β secondary structure in the intermediate state of the denaturation process (at 70 °C). For AK$_e$ and AKHC$_3$, the native CoH are conserved during the first thermal transition. Coupled with the CD data, the temperature-dependent NMR observations suggest that the parallel β-sheet and part of the surrounding α-helices are conserved in the intermediate state and denature only during the second thermal transition. Unfortunately, due to aggregation problems, the NMR study was limited to the first part of the denaturation process.

**Mechanism of the Thermal Unfolding**—The structural stability was further studied using urea as a chemical agent and CD spectroscopy. A single sigmoidal transition was observed for the two studied proteins (not shown). Curve fitting using a two-state model enabled us to calculate the thermodynamic parameters characterizing the structural stability of the proteins (Table I). The urea concentration at which half of the molecules are denatured ($C_m$) is significantly higher in AKC$_4$ variant reflecting a better resistance to urea denaturation of this protein. The calculated Gibbs free energy of unfolding, extrapolated to zero urea concentration (ΔG(H$_2$O)) is larger in the wild type relative to the engineered variant. Thus, despite an increased thermal stability and a higher resistance to chemical denaturation, the quadruple mutant has a lower structural stability in standard conditions (no denaturant, 20 °C). The coefficient of linear dependence of the Gibbs free energy of unfolding on the urea concentration ($m$) is considerably decreased in AKC$_4$, indicating a decreased cooperativity of unfolding in the engineered protein.

**DISCUSSION**

**Structure/Function Relationships**—As indicated by the CD and NMR results, introduction of 4 Cys residues and zinc binding in the engineered variant AKC$_4$ conserve the global fold of the polypeptide chain and induce only small structural changes in the LID domain. The Zn$^{2+}$-binding loops come closer to each other and the domain becomes more compact. The fact that the catalytic properties are practically conserved suggests that the domain keeps its ability to move toward the ATP-binding site. This is further supported by the fact that Ap$_5$A binding (which is known to favor this movement) increases the thermal unfolding mid-temperature ($T_m$) with similar extent in both wild type and AKC$_4$ (Fig. 4).

**Mechanism of the Thermal Unfolding**—The present experimental data provide a minimal structural basis for a basic description of the thermal unfolding process of AK$_e$. The CD experiments indicate a complex thermal denaturation mechanism with two separated cooperative steps. Comparative analysis of the NMR and CD data suggests that the protein core, including the parallel β-sheet and the surrounding helices (α1, α8, and α9), is conserved in the equilibrium intermediate state and unfolds in the second step. This domain represents about 45% of the total regular secondary structure (α-helix and β-sheet) contributing to the CD signal around 220 nm, in reasonable agreement with the relative extent of ellipticity change during the second unfolding transition (Fig. 4). In agreement with this hypothesis, Ap$_5$A shifts significantly the $T_m$ of the first transition but has only a minimal effect on the second one. Indeed, structural analysis of the bi-substrate binding (3) revealed that the most perturbed fragments are the connecting helices (α6 and α7) while the parallel β-sheet is not affected.

The present experiments provide no definite data on the role of the LID domain in the denaturation process. Recent $^{1}H^2$H exchange experiments on ^15N-labeled Ak$_e$ revealed that some amide protons in the antiparallel β-sheet exchange more rapidly than in the protein core (parallel β-sheet, α1, α8, and α9) but more slowly than in the peripheral α-helices. This suggests that the LID domain exhibits a more fluctuating hydrogen exchange pattern but does not play a role in unfolding.

---

TABLE I
Parameters of the thermal and urea unfolding for wild type and engineered adenylate kinases

| Protein   | AK$_e$ | ApoAKC$_4$ | AKC$_4$ |
|-----------|--------|------------|---------|
| A. Thermal unfolding |        |            |         |
| $T_m$ (°C) |        |            |         |
| ΔH$_{f}$ (kcal/mol) |        |            |         |
| ΔG$_{f}$ (H$_2$O) (kcal/mol) |        |            |         |
| m$^{c}$ (kcal/mol/°C) |        |            |         |

---

$^a$ $T_m$ is the mid-temperature of the first unfolding step, is the mean of two experiments. In parentheses, the estimated error of the given value.

$^b$ The parameters were obtained with a nonlinear least squares to fit the entire unfolding curve to a two-state model; the standard deviations are given in parentheses.

$^c$ Urea concentration at which half of the molecules are unfolded.

$^d$ The coefficient of linear dependence between the free energy of unfolding and urea concentration. $C_m$ is the urea concentration at which half of the molecules are unfolded.

---

$^{2}$ S. Burlacu-Miron and C. T. Craescu, unpublished results.
bond network than the parallel β-sheet and may unfold in early stages of denaturation. The large values of the temperature factors observed in x-ray studies in the LID domain of the unliganded AK, (4) support this hypothesis.

Interaction of AK with the bi-substrate Ap5A leads to a more symmetric and compact molecule and induces a structural stabilization of the nucleotide-closing domains (the antiparallel β-sheet, helices a2 and a3) and of the connecting helices (a6 and a7). The total accessible surface of the protein decreases by about 13% (4) and the ligand forms tight contacts with several side chains in the active site (20). These factors should contribute to the Ap5A-induced delay of the first denaturation step of the two proteins described here.

Increased Thermal Stability of AKC4—The experiments done on apoAKC4 indicate that the four side chain substitution and zinc binding have a cumulative effect in increasing the thermal stability. Analysis of the crystal structures suggests that the stability of the LID domain is mainly due to a network of hydrogen bond interactions between several side chains (including the 4 substituted residues, Arg131, Tyr133, and Glu151) keeping closer the two small β-sheets. The 4 Cys residues (by their smaller size and polar character) are able to integrate into this network, increasing the global thermal stability of the protein. Zn²⁺ binding has an additional contribution in restraining the global flexibility of this solvent-exposed domain. This decreased flexibility is one of the factors shown to differentiate the thermostable proteins from their mesophilic counterparts (21). By an induced effect, the zinc finger domain may also stabilize the helices connecting the domain to the protein core (a6 and a7), preventing the fraying of peripheral structural elements. In contrast, it has no significant effect on the unfolding of the parallel β-sheet which takes place at higher temperatures.

The $T_m$ of the AK from the thermophilic B. stearothermophilus (74.5 °C) (9) is similar to that of the second unfolding transitions reported here for AK$_e$ and AKC$_4$. This means that besides the contribution of the zinc binding region there are other contributions to the thermostability, especially related to the external helices. Indeed, when the metal ion is removed from the therophilic enzyme, $T_m$ decreases to 67 °C, a value which is still considerably higher than in AK$_e$.

In contrast with the increased stability against thermal and chemical denaturation of AKC$_4$, its structural stability in standard physicochemical conditions appears to be lower than that of the wild-type enzyme (Table I). The explanation of this observation requires a reliable thermodynamic characterization of the protein unfolding, based on combined variation of several physicochemical factors (temperature, pH, chemical denaturant, and so forth). Also, the complete NMR spectral assignment, the structure determination and characterization of the internal dynamics of the proteins will provide the necessary structural basis. These studies are currently in progress in our laboratory.

Acknowledgments—We thank Joel Mispelter and Petya Christova for useful discussions.

REFERENCES

1. Schulz, G. E. (1992) Curr. Opin. Struct. Biol. 2, 61–67
2. Schulz, G. E., Müller, C. W., and Biederhids, K. (1990) J. Mol. Biol. 213, 627–630
3. Gerstein, M., Schulz, G., and Chotia, C. (1993) J. Mol. Biol. 229, 494–501
4. Müller, C. W., Schlauderer, G. J., Reinstein, J., and Schulz, G. E. (1996) Structure 4, 147–156
5. Berry, M. B., Meador, B., Bilderback, T., Liang, P., Glaser, M., and Phillips, Jr., G. N. (1994) Proteins Struct. Funct. Genet. 19, 183–199
6. Vonrhein, C., Schlauderer, G. J., and Schulz, G. E. (1995) Structure 3, 483–490
7. Bilderback, T., Pulmer, T., Mantulin, W. W., and Glaser, M. (1996) Biochemistry 35, 6100–6106
8. Miyoshi, K., Eg, Y., Shioda, T., and Kawasaki, T. (1990) J. Biochem. 108,
Thermal Stability of Engineered Adenylate Kinase

267–270

9. Glaser, P., Presecan, E., Delepierre, M., Surewicz, W. K., Mantech, H. H., Bärzu, O., and Gilles, A.-M. (1992) Biochemistry 31, 3038–3043
10. Perrier, V., Burlacu-Miron, S., Bourgeois, S., Surewicz, W. K., and Gilles, A.-M. (1998) J. Biol. Chem. 273, 19097–19101
11. Pace, C. N. (1986) Methods Enzymol. 131, 266–280
12. Rance, M., Storersen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., and Wuthrich, K. (1983) Biochim. Biophys. Acta. 7479–7485
13. Kumar, A., Ernst, R. R., and Wuthrich, K. (1980) Biochim. Biophys. Acta. 595, 1–6
14. Davis, D. G., and Bax, A. (1985) J. Am. Chem. Soc. 107, 2820–2821
15. Johnson, Jr. C. W. (1990) Proteins Struct. Funct. Genet. 7, 205–214
16. Wuthrich, K. (1986) NMR of Proteins and Nucleic Acids, John Wiley & Sons, New York
17. Wishart, D. S., Sykes, B. D., and Richards, F. M. (1992) Biochemistry 31, 1647–1651
18. Monnot, M., Gilles, A.-M., Saint Giron, I., Michelson, S., Bärzu, O., and Fermandjian, S. (1987) J. Biol. Chem. 262, 2502–2506
19. Reinstein, J., Vetter, I. R., Schlichting, I., Roisch, P., Wittinghofer, A., and Goody, R. S. (1990) Biochemistry 29, 7440–7450
20. Müller, C. W., and Schulz, G. E. (1992) J. Mol. Biol. 224, 159–177
21. Macedo-Ribeiro, S., Darimont, B., Sterner, R., and Huber, R. (1966) Structure 4, 1291–1301
22. Kraulis, P. L. (1991) J. Appl. Crystallogr. 24, 946–950