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# Association of the catalytic subunit of aspartate transcarbamoylase with a zinc-containing polypeptide fragment of the regulatory chain leads to increases in thermal stability

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**Abstract**

The regulatory enzyme aspartate transcarbamoylase (ATCase), comprising 2 catalytic (C) trimers and 3 regulatory (R) dimers, owes its stability to the manifold interchain interactions among the 12 polypeptide chains. With the availability of a recombinant 70-amino acid zinc-containing polypeptide fragment of the regulatory chain of ATCase, it has become possible to analyze directly the interaction between catalytic and regulatory chains in a complex of simpler structure independent of other interactions such as those between the 2 C trimers, which also contribute to the stability of the holoenzyme. Also, the effect of the interaction between the polypeptide, termed the zinc domain, and the C trimer on the thermal stability and other properties can be measured directly. Differential scanning microcalorimetry experiments demonstrated that the binding of the zinc domain to the C trimer leads to a complex of markedly increased thermal stability. This was shown with a series of mutant forms of the C trimer, which themselves varied greatly in their temperature of denaturation due to single amino acid replacements. With some C trimers, for which $t_m$ varied over a range of 30 °C due to diverse amino acid substitutions, the elevation of $t_m$ resulting from the interaction with the zinc domain was as large as 18 °C. The values of $t_m$ for a variety of complexes of mutant C trimers and the wild-type zinc domain were similar to those observed when the holoenzymes containing the mutant C trimers were subjected to heat denaturation. In an extreme case with a mutant form involving replacement of Glu 86 by Ala in the catalytic chains, this was manifested by a change in $t_m$ for the trimer of 44.6 °C to 64.6 °C for the holoenzyme. These results contribute to our understanding of an earlier observation that scanning calorimetry on wild-type ATCase gave 2 transitions, with the high temperature peak, which is assigned to melting of C trimers, exhibiting a higher $t_m$ than isolated C trimer. The effect of the zinc domain on the $t_m$ of the complex with C trimer provides an explanation for this increase in thermal stability, i.e., during heat denaturation of the holoenzyme, the C trimer is still associated with the folded zinc domain fragments of regulatory chains.

**Keywords:** conformational change; differential scanning microcalorimetry; enhanced thermal stability; interchain interaction; zinc domain

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*Abbreviations:* ATCase, aspartate transcarbamoylase; C, catalytic trimer or subunit; R, regulatory dimer or subunit; c, catalytic polypeptide chain; r, regulatory polypeptide chain. Mutants with amino acid substitutions in the catalytic chain are denoted in the standard 1-letter code by the wild-type residue and numbered position in the polypeptide sequence, followed by the amino acid substitution. Thus, the replacement of Ser at position 52 by Gly is referred to as S52G. Mutant forms of the C trimer and ATCase are designated by subscripts, with wt representing wild type.

For the past 30 years most of the research on aspartate transcarbamoylase (ATCase; aspartate carbamoyltransferase, carbamoyl phosphate:L-aspartate carbamoyltransferase; EC 2.1.3.2) from *Escherichia coli* has been aimed toward an understanding of the allosteric transition whereby ATCase participates in the regulation of pyrimidine biosynthesis (Kantrowitz & Lipscomb, 1988; Schachman, 1988). More recently there has been considerable interest in structural features that provide stability of the enzyme while still permitting communication between the chains so that local binding events cause global effects throughout the entire molecule. These efforts have been only partially successful because of the complex dodecameric structure of the holoenzyme in which the 6 catalytic and 6 regulatory polypeptide chains in ATCase are organized as 2 catalytic trimers and 3 regulatory dimers through a multiplicity of diverse interchain interactions (Gerhart & Schachman, 1965; Weber, 1968a, 1968b; Wiley & Lipscomb, 1968; Rosenbusch & Weber, 1971; Cohlberg et al., 1972). Within each of the 2 C subunits are 3 sets of c:c contacts, and within each of the 3 R subunits are r:r contacts. In addition, each c chain in unliganded ATCase is linked to 2 different r chains, and the c chains from apposing C subunits interact with each other. Some of these interchain contacts are disrupted when ligands are bound at the active sites and the enzyme is converted from the low-affinity compact (**T**) state to the high-affinity, relaxed (**R**) state (Honzatko et al., 1982; Kim et al., 1987; Krause et al., 1987; Ke et al., 1988). How the changes in these interactions mediate the allosteric transition has been the subject of much investigation (Subramani & Schachman, 1980; Eisenstein et al., 1989; Newell & Schachman, 1990; Tauc et al., 1990; Newton et al., 1992).

Because of the complications inherent in the multiple interactions and the changes that occur upon the binding of ligands, it has been difficult to delineate the role of any single contact in modulating the conformational changes and in modifying the properties of the C and R subunits when they are incorporated into the holoenzyme. Considerable simplification of the problem has been achieved by the isolation of a 70-amino acid zinc-containing polypeptide fragment from the r chain (Markby et al., 1991). This polypeptide, termed the zinc domain, was shown to form a stable complex with the C trimer, leading to marked changes in the functional properties of the enzyme (Zhou & Schachman, 1993 [including kinemage file]). In this complex the subset of interactions at the c:r interface can be analyzed apart from the myriad other interchain interactions that contribute to the stability of the dodecameric holoenzyme.

In this study, the effect of the binding of the zinc domain to the C subunit is illustrated by the greatly enhanced thermal stability of the complex. The results also provide an explanation for earlier perplexing results of differential scanning microcalorimetry experiments on ATCase (Vickers et al., 1978; Edge et al., 1988; Peterson & Schachman, 1991). The thermogram for wild-type ATCase shows 2 partially overlapping transitions, with that at the lower temperature attributed to the melting of the R subunits and the endotherm at the higher temperature to the C subunits. As expected from energetic coupling between subunits (Brandts et al., 1989), the $t_m$ assigned to the R subunit in ATCase is significantly higher than the measured $t_m$ of the isolated R dimer. However, the $t_m$ ascribed to the C trimers in ATCase also appeared to be significantly greater than that observed for the purified C trimer. Because the R subunits were presumed to be already "melted" before the transition temperature for the C trimer was attained, it seemed unlikely that energetic coupling between the denatured r chains and still native C trimers could have much effect. Alternatively, there was the possibility that only part of each r chain denatures in the first thermal transition, leaving part of each chain folded and bound to the C trimers through interchain interactions. Having the isolated zinc domain available provides a useful probe for analyzing the differential scanning microcalorimetry experiments on wild-type ATCase and various mutant forms containing amino acid substitutions in the c chains. As shown here, formation of a stable complex between the 70-amino acid zinc domain and C trimer leads to a substantial increase in thermal stability.

## Results

### *Mutant forms of C subunit and holoenzyme differ markedly in thermal stability*

Single amino acid replacements in various positions of the c chain have a marked effect on the thermal stability of the C trimer, with some substitutions leading to a 13 °C increase in the melting temperature and others to a 15 °C decrease. As seen in Figure 1A, $C_{wt}$ exhibits a broad, asymmetric endotherm with a $t_m$ of 60.9 °C (Edge et al., 1985; Peterson & Schachman, 1991). Calorimetric experiments on 2 mutant forms of the C trimer, which are representative of extreme alterations in thermal stability arising from single amino acid replacements, are shown in Figure 1B and C. The $C_{R65M}$ trimer exhibits a marked destabilization to thermal denaturation (Fig. 1B) manifested by a sharper, more symmetrical melting curve with a $t_m$ of 45.6 °C. In contrast, $C_{S52C}$ is significantly more stable than $C_{wt}$; the thermogram in Figure 1C shows a sharp, almost symmetrical melting curve with a $t_m$ of 74.2 °C.

The effects of the amino acid substitutions are also manifested in the differential scanning microcalorimetry experiments on the holoenzymes. As seen in Figure 1A, $ATCase_{wt}$ denatures in 2 distinct but overlapping phases with 1 transition at 65 °C and the second at 69 °C. The first of these endotherms has been attributed previously to denaturation of the R subunits, and the second with the higher $t_m$ to the melting of the C subunits (Vickers et al., 1978; Edge et al., 1988; Peterson & Schachman, 1991). In thermal denaturation experiments on various mutant forms of ATCase and C trimer, several mutant holoenzymes exhibited properties similar in many respects to the wild-type enzyme. These are termed Wild-type-like[4] and results on some are listed in Table 1. For each of these enzymes, the second endotherm occurred at a higher temperature than the $t_m$ observed for the isolated C trimer. However, the difference between the 2 values, $\Delta t_m$, evaluated from the data, varied considerably from 10.9 °C for $ATCase_{S52G}$ to only 1.0 °C for $ATCase_{R296A}$.

[4] The division of the various mutant forms of the C trimers of ATCase into 3 classes is somewhat arbitrary. There is some tendency for overlap, and the classification is based both on the $t_m$'s of the mutant C trimers and on the denaturation characteristics of the holoenzymes. Those holoenzymes in the class Wild-type-like showed 2 endotherms, as did those in the class More Stable. This latter class was designated More Stable because the isolated C trimers denatured with $t_m$'s at least 10 °C higher than wild-type C trimer. Those mutants designated Less Stable not only had much lower $t_m$'s for the C trimers than for $C_{wt}$, but also exhibited only a single endotherm in calorimetry experiments on the holoenzymes.

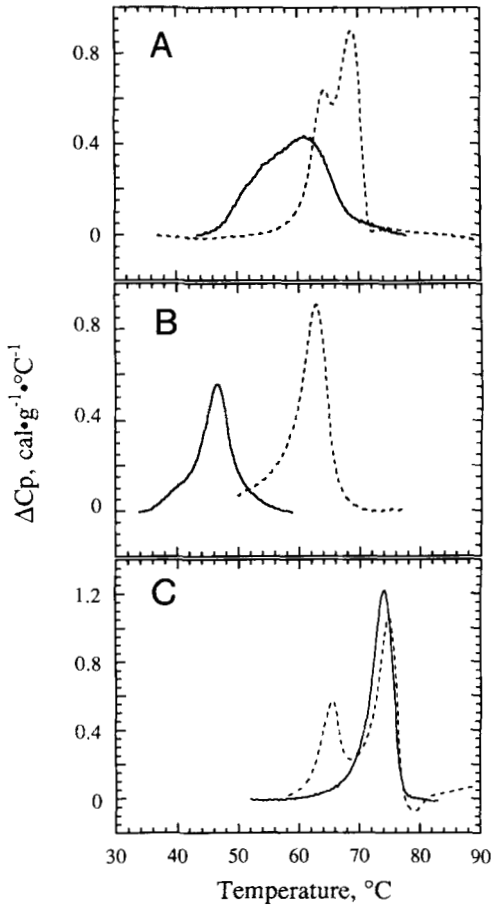


**Fig. 1.** Differential scanning calorimetry of wild-type and mutant forms of C trimer and of ATCase holoenzyme. Scans were performed at a rate of 53 °C per hour in a borate buffer, as described in Materials and methods. **A:** Plots of the observed increase in heat capacity (ΔCp) vs. temperature are shown by the solid line for wild-type C trimer (2.29 mg/mL) and by the broken line for wild-type holoenzyme (3 mg/mL) (taken from Peterson & Schachman, 1991). **B:** Thermogram for Less Stable C trimer, $C_{R65M}$ (solid line), at 2.13 mg/mL along with the thermogram for $ATCase_{R65M}$ (broken line) at 2.83 mg/mL. **C:** Thermogram for the More Stable C trimer, $C_{S52C}$ (solid line) at 2.38 mg/mL and for $ATCase_{S52C}$ (broken line) at 3.66 mg/mL.

**Table 1.** *Thermal denaturation of C trimers and holoenzymes*

| Class of enzyme | Amino acid substitution | $t_m$ (°C) | | $\Delta t_m$[b] (°C) |
|---|---|---|---|---|
| | | C trimer | Holoenzyme[a] | |
| Wild-type-like | S52G | 58.1 | 65.0, 69.0 | 10.9 |
| | Wild-type | 60.9 | 65.0, 69.0 | 8.1 |
| | Q231I | 62.5 | 58.8, 64.8 | 2.3 |
| | R296A | 64.0 | 58.0, 65.0 | 1.0 |
| | N78D | 67.3 | 64.8, 71.2 | 3.9 |
| Less Stable | E86A | 44.6 | 64.6 | 20.0 |
| | R65M | 45.6 | 63.0 | 17.4 |
| | S52T | 48.0 | 65.0 | 17.0 |
| | L277I | 52.3 | 62.8 | 10.5 |
| | G128D | 53.0 | 59.6 | 6.6 |
| More Stable | S52A | 71.1 | 64.1, 70.5 | −0.6 |
| | S52C | 74.2 | 65.3, 74.8 | 0.6 |
| | R54H | 74.2 | 65.0, 75.0 | 0.8 |

[a] A single $t_m$ is listed for holoenzymes in the Less Stable class; they all exhibited single endotherms. For the holoenzymes that exhibit 2 endotherms, $t_m$'s for both peaks are listed.

[b] $\Delta t_m$ represents the difference between the $t_m$ observed for the C subunit in the holoenzyme and that of the isolated C trimer. For holoenzymes that exhibit 2 peaks, this is the $t_m$ for the higher temperature transition.

For this class of mutant holoenzymes the $t_m$ representing the lower temperature transition was significantly higher than the $t_m$ for isolated R subunits, which was 54.4 °C under the same conditions (Peterson & Schachman, 1991). Those mutants such as $ATCase_{Q231I}$ and $ATCase_{R296A}$, which exhibited small values of $\Delta t_m$ for the C trimers when incorporated into holoenzymes, also showed much smaller increases in $t_m$ for the R subunits in the holoenzymes as compared to isolated dimers.

Some mutant forms were significantly less stable than wild-type C trimer, and five of them are designated in Table 1 as Less Stable. The holoenzymes containing mutant C trimers in the Less Stable class exhibited single endotherms in differential scanning microcalorimetry experiments. This result is seen for $ATCase_{R65M}$ in Figure 1B, and the data for the others are summarized in Table 1. For these mutant holoenzymes, the values of $\Delta t_m$, though varying for the different amino acid substitutions, are much higher than the corresponding values in the other 2 classes.

The third group, including $C_{S52C}$ (Fig. 1C), is designated in Table 1 as More Stable. Like the holoenzymes in the Wild-type-like class, 2 endotherms were observed for those holoenzymes containing More Stable C trimers. For these mutant forms of ATCase there was virtually no increase in the $t_m$ attributed to the C trimer upon its incorporation into the holoenzyme.

In addition to the substantial variations in $\Delta t_m$ represented by the data in Table 1, it is striking that the mutant trimers, each containing only a single amino acid substitution, themselves encompass a range of $t_m$ values of about 30 °C. Also, the replacement at a single locus, Ser 52, by 4 different amino acids yields trimers with $t_m$'s varying by 26 °C.

### *Binding of zinc domain to C trimer leads to a complex of greatly enhanced thermal stability*

Because the C subunits in wild-type ATCase appear to denature at a temperature about 8 °C higher than the free C trimer (Table 1; Fig. 1A), it seems that incorporation of the C subunits, along with R subunits, into the holoenzyme leads to enhanced thermal stability. This result seems paradoxical in that the low temperature transition in the melting of ATCase is almost certainly attributable to denaturation of the R subunits. If the holoenzyme structure is disrupted by the melting of the R subunits, why do not the C subunits denature at 60.9 °C as they would in experiments on the free subunits? Is there a segment of each r chain that is not denatured at 65 °C and remains tightly associated with each of the c chains in a C trimer, thereby leading to enhanced thermal stability of the putative complex? This pos-

sibility was tested by calorimetry experiments on mixtures of the zinc domain and various mutant C trimers.

As seen in Figure 2A, the addition of zinc domain to $C_{wt}$ trimer (at a 1.7 molar ratio in terms of polypeptide chains) results in a marked increase in thermal stability with $t_m$ increasing from 60.9 °C for the $C_{wt}$ trimer to 72.6 °C for the complex. In addition, the endotherm was much sharper. A mutant form from the Less Stable class is illustrated by the experiment with $C_{R65M}$ trimer shown in Figure 2B. For this mutant trimer the formation of a complex with the zinc domain leads to a $\Delta t_m$ of 14.6 °C. With $C_{G128D}$ trimer, another mutant form in the Less Stable class, the change in $t_m$ upon interaction with the zinc domain is 8 °C (from 53 °C to 61 °C), whereas the mutant holoenzyme has a $t_m$ of 59.6 °C corresponding to a $\Delta t_m$ of 6.6 °C. In contrast to the effects of the zinc domain on these C trimers, only a small increase in stability was observed upon the formation of the complex between the zinc domain and $C_{R54H}$ trimer, a member of the More Stable class. The endotherms for the free $C_{R54H}$ trimer and the complex are shown in Figure 2C, and the results for all of the mutants examined thus far are summarized in Table 2.

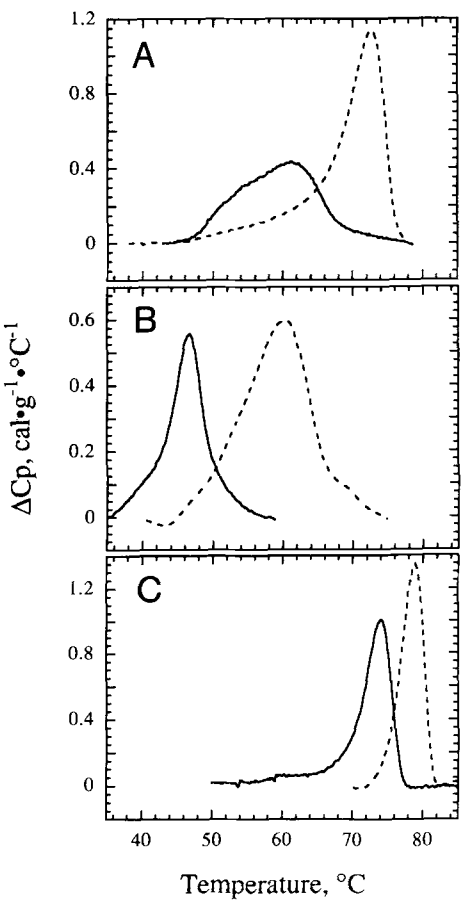


**Fig. 2.** Thermograms for complexes of the zinc domain with wild-type and mutant forms of the C trimer. **A:** Thermogram for the complex of zinc domain with wild-type C subunit is shown by the broken line, with the scan for the isolated C trimer (reproduced from Fig. 1A) in the solid line. $C_{wt}$ at a concentration of 3 mg/mL was mixed with zinc domain at a final concentration of 1.3 mg/mL, to give a molar ratio of zinc domain to C trimer of 5:1. **B:** Representative plots of excess heat capacity vs. temperature for the Less Stable $C_{R65M}$ (solid line, reproduced from Fig. 1B) and for the complex between the zinc domain polypeptide and $C_{R65M}$ (broken line). The concentration of $C_{R65M}$ was 1 mg/mL, and the final concentration of added zinc domain was 3 mg/mL, so that the zinc domain polypeptide was present in a 32-fold molar excess. **C:** A calorimetric scan representing the complex of zinc domain with the More Stable $C_{R54H}$ is shown by the broken line, along with the scan for the $C_{R54H}$ trimer alone in the solid line. Protein concentration was 3 mg/mL for $C_{R54H}$, with a final concentration of 1.3 mg/mL zinc domain, to give a 5:1 ratio of zinc domain to $C_{R54H}$ trimer.

These data for the enhanced thermal stability of the complexes of C trimers and the zinc domain indicate that the contacts between the trimer and the polypeptide are sufficient to account for most of the increase in stability when C trimers are incorporated into holoenzymes. For $C_{R65M}$ trimer the change in $t_m$ upon the interaction with the zinc domain is 14.6 °C (from 45.6 °C to 60.2 °C), whereas the mutant holoenzyme has a $t_m$ of 63.0 °C, corresponding to a $\Delta t_m$ of 17.4 °C.

One mutant trimer, $C_{R65K}$, differed so strikingly from the others that it could not easily be categorized in any of the 3 classes summarized in Table 1. Differential scanning microcalorimetry on $C_{R65K}$ trimer yielded a single endotherm (Fig. 3A) with a $t_m$ of 51 °C. By this criterion, the mutant trimer could be placed in the Less Stable class. However, as a holoenzyme, $ATCase_{R65K}$ exhibited 2 partially overlapping transitions with $t_m$'s of 64.4 °C and 67.8 °C. In this respect the mutant holoenzyme would be placed in the Wild-type-like class despite the fact that the $t_m$ of $C_{R65K}$ trimer is much less than that of $C_{wt}$ trimer as well as that of $R_{wt}$ dimer. The value of $\Delta t_m$ for the incorporation of the trimer into the holoenzyme is 16.8 °C. In order to determine whether the interactions with the zinc domain could account for this enhanced stabilization, we performed thermal denaturation experiments on the complex between $C_{R65K}$ and the zinc domain. As seen in Figure 3B and Table 2, there was a marked increase in thermal stability corresponding to a $\Delta t_m$ of 17.4 °C. Thus, the interaction of the trimer with the polypeptide has the same effect on the thermal stability of the complex as incorporating the trimers in holoenzymes.

### *Binding of one zinc-containing polypeptide to a c chain may influence subsequent binding to other chains in the same C trimer*

Earlier studies on C subunits (Kempe & Stark, 1975; Lahue & Schachman, 1986) have provided evidence that there is "communication" between adjacent chains in a trimer. It was of interest, therefore, to determine whether the binding of 1 zinc domain to a trimer would result in a conformational change

**Table 2.** *Effect of zinc domain on thermal stability of mutant C trimers*

| Amino acid substitution | $t_m$ (°C) | | $\Delta t_m$ (°C) |
|---|---|---|---|
| | C trimer | Complex | |
| Wild type | 60.9 | 72.6 | 11.7 |
| G128D | 53.0 | 61.0 | 8.0 |
| R65M | 45.6 | 60.2 | 14.6 |
| R54H | 74.2 | 78.8 | 4.6 |
| R65K | 51.0 | 68.4 | 17.4 |

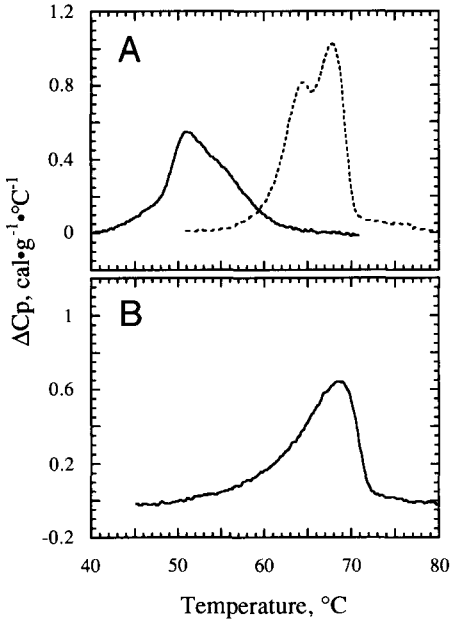


**Fig. 3.** Calorimetric scans for $ATCase_{R65K}$, $C_{R65K}$ trimer, and complex of the $C_{R65K}$ trimer with the zinc domain polypeptide. **A:** Thermograms for $C_{R65K}$ (1.55 mg/mL, solid line) and the R65K holoenzyme (2.5 mg/mL, broken line). **B:** Thermogram for the zinc domain–$C_{R65K}$ trimer complex. $C_{R65K}$ trimer was at a concentration of 1.59 mg/mL and zinc domain was at a concentration of 0.7 mg/mL so that zinc domain was present in the mixture in a 5-fold molar excess.

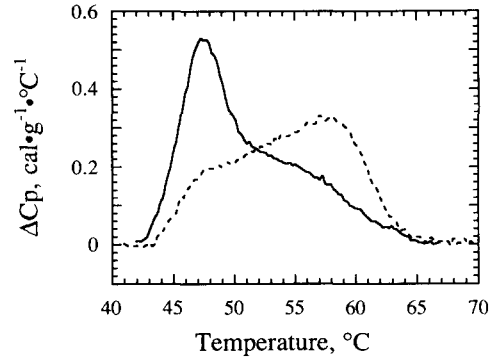


**Fig. 4.** Thermograms of mixtures of the $C_{R65M}$ with 2 different subsaturating concentrations of the zinc domain. The solid line represents data obtained by mixing $C_{R65M}$ with the zinc-binding polypeptide in a molar ratio of 1:2.5, and the broken line shows results at a 1:5 molar ratio. Even a 5-fold molar excess of zinc domain over C trimer was not saturating due to the weaker nature of c:r interactions with the mutant form of this enzyme. Protein concentration for the $C_{R65M}$ trimer was 1.47 mg/mL, with zinc domain at a concentration of 0.32 (solid line) and 0.64 mg/mL (broken line). These data can be compared with the thermogram for $C_{R65M}$ fully saturated with the zinc domain as shown in Figure 2B.

throughout the molecule, thereby facilitating binding of the polypeptide at the other 2 sites. If the 2 species interact strongly, such cooperativity in binding should be detectable in differential scanning microcalorimetry experiments on mixtures of C trimers with substoichiometric amounts of zinc domain. At one extreme we would observe an endotherm for free C trimer and a second, at a higher temperature, corresponding to the complex of C trimer and 3 zinc domains. If, on the contrary, the binding of the polypeptide to each of the 3 sites on the C trimer is independent, one would *not* expect a biphasic endotherm for mixtures of C trimer and substoichiometric amounts of zinc domain.

Experiments with $C_{wt}$ trimer and substoichiometric amounts of zinc domain were inconclusive largely because the endotherm of free $C_{wt}$ trimer is so broad and asymmetric (Fig. 1A) and the $\Delta t_m$ resulting from saturation with zinc domain is only 11.7 °C. Accordingly, preliminary experiments were performed with $C_{R65M}$ trimer and different amounts of zinc domain. In some respects this system is preferable to that using $C_{wt}$ trimer because the $\Delta t_m$ resulting from binding zinc domain is almost 15 °C. Also, the endotherm for the free mutant trimer is much sharper. Hence, a biphasic endotherm would be easier to resolve, and complexes with intermediate thermal stabilities would be more readily detectable. This system has a major disadvantage, however, because the interaction of the zinc domain with $C_{R65M}$ trimer is much weaker than with $C_{wt}$ trimer. Thus, for the differential scanning microcalorimetry experiment illustrated in Figure 4, a molar excess of zinc-containing polypeptide per c chain was required. When the ratio was only 2.5:1, the endotherm showed that free $C_{R65M}$ subunit is the dominant species with a shoulder representing minor species with a higher $t_m$. Increasing the ratio of zinc domain per c chain to 5:1 led to the opposite situation with the predominant species having a $t_m$ of at least 59 °C, close to that obtained for $ATCase_{R65M}$. The endotherm for this mixture also showed material that appeared to have a $t_m$ of free $C_{R65M}$ trimer (46 °C). Although the presence of complexes with intermediate $t_m$'s cannot be ruled out, the endotherms in Figure 4 do indicate that binding of zinc domain to C trimers may be cooperative.

## Discussion

As seen in Table 1, single amino acid substitutions in diverse regions of the c polypeptide chains of ATCase cause striking changes in the stability of the C trimers. Some replacements led to a substantial reduction in $t_m$ of 15 °C compared to $C_{wt}$ trimer ($t_m$ = 60.9 °C), whereas other substitutions resulted in enhanced thermal stability represented by a 13 °C increase in $t_m$. Moreover, 4 different replacements of S52 yielded a series of mutant trimers with $t_m$'s encompassing a range from 48 to 74 °C. It is not our purpose here to focus on these dramatic changes in stability in terms of the locations of the specific amino acid residues and the effects of the various substitutions on packing, cavity formation, ionic interactions, or alterations in hydrophobicity. Even with the exquisite structural detail that has been provided for this enzyme (Honzatko et al., 1982; Kim et al., 1987; Krause et al., 1987; Ke et al., 1988), it is difficult to predict changes in stability resulting from the various amino acid substitutions. Rather, our goal is to understand the effects on the various mutant trimers stemming from their interactions with R subunits upon their assembly into holoenzymes resembling $ATCase_{wt}$.

With the exception of a group of mutant trimers in the More Stable class (Table 1), all of which had $t_m$ values about 71 °C, the incorporation of trimers into ATCase-like holoenzymes led to a significant increase in $t_m$. For those mutant trimers that

had a $t_m$ similar to that of $C_{wt}$, the values of the increase in the *apparent* denaturation temperature of the C subunit in the holoenzyme compared to the $t_m$ for the free C trimer varied from 1 to 10 °C. In contrast, these values for $\Delta t_m$ were as large as 20 °C for some of the mutant trimers in the Less Stable class (Table 1). Two of the classes of mutant holoenzymes exhibited biphasic endotherms, with that at the lower temperature having values of $t_m$ ranging from 58 to 65 °C. This endotherm at the lower temperature has been attributed to the denaturation of the R subunits in the holoenzyme (Vickers et al., 1978; Edge et al., 1988; Burz et al., 1990; Peterson & Schachman, 1991). The increase in $t_m$ for the denaturation of the R subunits in the holoenzyme compared to isolated R dimers, for which $t_m$ is 54.4 °C, has been attributed to energetic coupling between the C and R subunits (Brandts et al., 1989). In many of the mutants the interchain interactions are so strong that the apparent denaturation temperature for the denaturation of the R subunits in the holoenzyme is more than 10 °C higher than that for free R dimers. For 2 mutant forms of the enzyme, $ATCase_{R296A}$ and $ATCase_{Q231I}$, which have been shown to have markedly weakened interactions between the c and r chains (Peterson et al., 1992; Peterson & Schachman, 1992), the increase in $t_m$ for the apparent denaturation of the R subunits in the holoenzyme compared to free R dimers is only 4–5 °C, in accord with the concept of energetic coupling as proposed by Brandts et al. (1989).

Although the apparent enhanced stability of the R subunits in ATCase is readily explained as the result of the interaction of those subunits with C subunits, no such explanation can account satisfactorily for the values of $\Delta t_m$ for the apparent denaturation of the C subunits in the holoenzymes as compared to free C trimers (Table 1). If the R subunits had already been "melted," then energetic coupling between the still folded chains of intact C trimers and the denatured r chains would be negligible, and the $t_m$ for the C subunit would be expected to be essentially equal to that for free C trimer. It is clear now from the differential scanning microcalorimetry on the complexes between the mutant C trimers and the zinc domain (Table 2) that the previous assignments of the low temperature endotherm to denaturation of the R subunit and, at the higher temperature (Fig. 1A) to the C subunit, must be modified. Because it is evident that the complex between C trimer and zinc domain has a higher $t_m$ than the free C subunit, we can attribute the higher temperature endotherm observed with the holoenzyme not to C subunits alone but rather to the complex between those subunits and the zinc domain segment of the r chain. Similarly, the endotherm at the lower temperature would represent the denaturation of only part of the R subunits, i.e., the region of the r chain representing the allosteric domain that forms the interchain contacts responsible for the formation and stability of R dimers.

The $\Delta t_m$ observed for C subunit upon its incorporation into holoenzyme (Fig. 1; Table 1) is exaggerated under the conditions used in the present experiments compared to earlier studies (Vickers et al., 1978). For wild-type enzyme, $\Delta t_m$ is approximately 8 °C in experiments with borate buffer at pH 9.0 and a scan rate near 1 °K/min, whereas $\Delta t_m$ observed previously (Vickers et al., 1978) was only 2 °C for enzyme in a phosphate buffer at pH 7.0 and a more rapid scan rate. Also, the calorimetric studies of Edge et al. (1985, 1988) on the holoenzyme and C subunit using phosphate buffer at pH 7.0, with a scan rate of only 0.22 °K/min, exhibit no $\Delta t_m$ for the C subunit upon assembly into the holoenzyme. A major factor accounting for the different results is the presence of phosphate, which is a product of the reaction catalyzed by ATCase and which binds at the active sites. Phosphate binding to C trimer would be expected to result in a complex of increased stability. Accordingly, the $t_m$'s for both free C trimer and the C subunits incorporated into ATCase are higher in phosphate buffer than in either the borate buffer used in this work or in an imidazole buffer (Vickers et al., 1978). It is worth noting that there is a dependence of $t_m$ on the concentration of phosphate (Vickers et al., 1978). In view of the diverse effects that ligands have on the strengths of interaction at the multiple interfaces among the 12 polypeptide chains in the holoenzyme (Subramani et al., 1977; Subramani & Schachman, 1980; Yang & Schachman, 1987), it is difficult to evaluate the effect of phosphate on only the c:r interactions. The study of the stability of the complex of C trimer with the zinc domain should facilitate such investigations.

## Materials and methods

Replacements of amino acid residues in the c chain of ATCase were performed by oligonucleotide-directed mutagenesis, using mutagenic primers (approximately 20 bases in length) synthesized on an Applied Biosystems 380B synthesizer (Eisenstein et al., 1989). The mutant forms of the protein were identified by nucleotide sequence determination (Sanger et al., 1977). The mutant form of ATCase that contains Asp in place of Gly at position 128 in the c chain was generated originally by random mutagenesis and has been characterized extensively (Wall et al., 1979; Wall & Schachman, 1979). Wild-type and mutant forms of ATCase were overproduced in *Escherichia coli* strain EK1104 grown in minimal medium supplemented as described elsewhere (Nowlan & Kantrowitz, 1985). Holoenzymes were purified from the cell extract using the procedures described with minor modifications. Mercurial reagents were used to dissociate the holoenzymes into C and R subunits, and free C subunits were isolated by ion-exchange chromatography on a DEAE matrix (Yang et al., 1978).

The 70-amino acid polypeptide, corresponding to the zinc domain of the r chain of ATCase, was expressed and purified as described previously (Markby et al., 1991; Zhou & Schachman, 1993). Concentrations of the polypeptide were determined spectrophotometrically using an extinction coefficient of 0.32 mL/mg/cm. Complex formation between the zinc domain and C trimer was demonstrated by a shift in electrophoretic migration of the complex compared to free C subunit or the isolated zinc domain using 7.5% polyacrylamide gels in a nondenaturing buffer system (Jovin et al., 1964).

Differential scanning calorimetry was performed on a Microcal MC-2 instrument interfaced with an IBM-XT computer for data collection and analysis. The instrument was operated at a scan rate of 53 °C per h in the temperature range 30–90 °C. Buffer for the experiments was 40 mM potassium borate, pH 9.0, containing 5 mM β-mercaptoethanol and 0.2 mM EDTA (Peterson & Schachman, 1991). Calorimetric measurements on mixtures containing purified zinc domain also contained 0.5 mM zinc acetate in the solution. Sample concentrations were 1–3 mg/mL for C trimer and 2–4 mg/mL for holoenzymes. The concentration of zinc domain polypeptide varied according to the stoichiometry of zinc domain to C trimer desired for the experiment and the strength of the interaction between the various mutant C trimers and zinc domain. Assignment of $t_m$'s in ther-

mograms for the several forms of ATCase characterized in this study, the corresponding C trimers, and complexes between the C trimer and zinc domain were made at the temperature at which the maximum increase in heat capacity above baseline levels is observed.

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## References

Brandts JF, Hu CQ, Lin LN, Mas MT. 1989. A simple model for proteins with interacting domains. Applications to scanning calorimetry data. *Biochemistry 28*:8588–8596.

Burz DS, Allewell NM, Ghosaini L, Hu CQ, Sturtevant JM. 1990. Differential scanning calorimetric studies of *E. coli* aspartate transcarbamylase. *Biophys Chem 37*:31–41.

Cohlberg JA, Pigiet VP Jr, Schachman HK. 1972. Structure and arrangement of the regulatory subunits in aspartate transcarbamylase. *Biochemistry 11*:3396–3411.

Edge V, Allewell NM, Sturtevant JM. 1985. High-resolution differential scanning calorimetric analysis of the subunits of *E. coli* aspartate transcarbamylase. *Biochemistry 24*:5899–5906.

Edge V, Allewell NM, Sturtevant JM. 1988. Differential scanning calorimetric study of the thermal denaturation of aspartate transcarbamoylase of *Escherichia coli*. *Biochemistry 27*:8081–8087.

Eisenstein E, Markby DW, Schachman HK. 1989. Changes in stability and allosteric properties of aspartate transcarbamoylase resulting from amino acid substitutions in the zinc-binding domain of the regulatory chains. *Proc Natl Acad Sci USA 86*:3094–3098.

Gerhart JC, Schachman HK. 1965. Distinct subunits for the regulation and catalytic activity of aspartate transcarbamylase. *Biochemistry 4*:1054–1062.

Honzatko RB, Crawford JL, Monaco HL, Ladner JE, Edwards BFP, Evans DR, Warren SG, Wiley DC, Ladner RC, Lipscomb WN. 1982. Crystal and molecular structures of native and CTP liganded aspartate carbamoyltransferase. *J Mol Biol 160*:219–263.

Jovin T, Chrambach A, Naughton MA. 1964. An apparatus for preparative temperature-regulated polyacrylamide gel electrophoresis. *Anal Biochem 9*:351–369.

Kantrowitz ER, Lipscomb WN. 1988. *Escherichia coli* aspartate transcarbamylase: The relation between structure and function. *Science 241*:669–674.

Ke H, Lipscomb WN, Cho Y, Honzatko RB. 1988. Complex of *N*-phosphonacetyl-L-aspartate with aspartate carbamoyltransferase. X-ray refinement, analysis of conformational changes and catalytic and allosteric mechanisms. *J Mol Biol 204*:724–747.

Kempe TD, Stark GR. 1975. Pyridoxal 5′-phosphate, a fluorescent probe in the active site of aspartate transcarbamylase. *J Biol Chem 250*:6861–6869.

Kim KH, Pan Z, Honzatko RB, Ke HM, Lipscomb WN. 1987. Structural asymmetry in the CTP-liganded form of aspartate carbamoyltransferase from *Escherichia coli*. *J Mol Biol 196*:853–875.

Krause KL, Volz KW, Lipscomb WN. 1987. 2.5 Å structure of aspartate carbamoyltransferase complexed with the bisubstrate analog *N*-(phosphonacetyl)-L-aspartate. *J Mol Biol 193*:527–553.

Lahue RS, Schachman HK. 1986. Communication between polypeptide chains in aspartate transcarbamoylase conformational changes at the active sites of unliganded chains resulting from ligand binding to other chains. *J Biol Chem 261*:3079–3084.

Markby DW, Zhou BB, Schachman HK. 1991. A 70-amino acid zinc-binding polypeptide from the regulatory chain of aspartate transcarbamoylase forms a stable complex with the catalytic subunit leading to markedly altered enzyme activity. *Proc Natl Acad Sci USA 88*:10568–10572.

Newell JO, Schachman HK. 1990. Amino acid substitutions which stabilize aspartate transcarbamoylase in the R state disrupt both homotropic and heterotropic effects. *Biophys Chem 37*:183–196.

Newton CJ, Stevens RC, Kantrowitz ER. 1992. Importance of a conserved residue, aspartate-162, for the function of *Escherichia coli* aspartate transcarbamoylase. *Biochemistry 31*:3026–3032.

Nowlan SF, Kantrowitz ER. 1985. Superproduction and rapid purification of *E. coli* aspartate transcarbamylase and its catalytic subunit under extreme derepression of the pyrimidine pathway. *J Biol Chem 260*:14712–14716.

Peterson CB, Burman DL, Schachman HK. 1992. Effects of replacement of active site residue glutamine 231 on activity and allosteric properties of aspartate transcarbamoylase. *Biochemistry 31*:8508–8515.

Peterson CB, Schachman HK. 1991. Role of a carboxyl-terminal helix in the assembly, interchain interactions, and stability of aspartate transcarbamoylase. *Proc Natl Acad Sci USA 88*:458–462.

Peterson CB, Schachman HK. 1992. Long range effects of amino acid substitutions in the catalytic chain of aspartate transcarbamoylase. Localized replacements in the carboxyl-terminal α-helix cause marked alterations in allosteric properties and intersubunit interactions. *J Biol Chem 267*:2443–2450.

Rosenbusch JP, Weber K. 1971. Subunit structure of aspartate transcarbamylase from *Escherichia coli*. *J Biol Chem 246*:1644–1657.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA 74*:5463–5467.

Schachman HK. 1988. Can a simple model account for the allosteric transition of aspartate transcarbamoylase? *J Biol Chem 263*:18583–18586.

Subramani S, Bothwell MA, Gibbons I, Yang YR, Schachman HK. 1977. Ligand-promoted weakening of intersubunit bonding domains in aspartate transcarbamoylase. *Proc Natl Acad Sci USA 74*:3777–3781.

Subramani S, Schachman HK. 1980. Mechanism of disproportionation of aspartate transcarbamoylase molecules lacking one regulatory subunit. *J Biol Chem 255*:8136–8143.

Tauc P, Vachette P, Middleton SA, Kantrowitz ER. 1990. Structural consequences of the replacement of Glu 239 by Gln in the catalytic chain of *Escherichia coli* aspartate transcarbamylase. *J Mol Biol 214*:327–335.

Vickers LP, Donovan JW, Schachman HK. 1978. Differential scanning calorimetry of aspartate transcarbamoylase and its isolated subunits. *J Biol Chem 253*:8493–8498.

Wall KA, Flatgaard JE, Gibbons I, Schachman HK. 1979. Purification and characterization of a mutant aspartate transcarbamoylase lacking enzyme activity. *J Biol Chem 254*:11910–11916.

Wall KA, Schachman HK. 1979. Primary structure and properties of an inactive mutant aspartate transcarbamoylase. *J Biol Chem 254*:11917–11926.

Weber K. 1968a. Aspartate transcarbamylase from *Escherichia coli*. Characterization of the polypeptide chains by molecular weight, amino acid composition, and amino-terminal residues. *J Biol Chem 243*:543–546.

Weber K. 1968b. New structural model of *E. coli* aspartate transcarbamylase and the amino-acid sequence of the regulatory polypeptide chain. *Nature (Lond) 218*:1116–1119.

Wiley DC, Lipscomb WN. 1968. Crystallographic determination of symmetry of aspartate transcarbamylase. *Nature (Lond) 218*:1119–1121.

Yang YR, Kirschner MW, Schachman HK. 1978. Aspartate transcarbamoylase *Escherichia coli*: Preparation of subunits. *Methods Enzymol 51*:35–41.

Yang YR, Schachman HK. 1987. Hybridization as a technique for studying interchain interactions in the catalytic trimers of aspartate transcarbamoylase. *Anal Biochem 163*:188–195.

Zhou BB, Schachman HK. 1993. Peptide–protein interaction markedly alters the functional properties of the catalytic subunit of aspartate transcarbamoylase. *Protein Sci 2*:103–112.