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Heterotropic interactions in aspartate transcarbamoylase: Turning allosteric ATP activation into inhibition as a consequence of a single tyrosine to phenylalanine mutation

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ABSTRACT **Aspartate transcarbamoylase (EC 2.1.3.2) is extensively studied as a model for cooperativity and allostery. This enzyme shows cooperativity between the catalytic sites, and its activity is feedback inhibited by CTP and activated by ATP. These regulatory processes involve several interfaces between catalytic and regulatory chains as well as between domains within these two types of chains. As far as the regulatory chain is concerned, its two domains are in contact through a hydrophobic interface, in which a tyrosine residue is inserted in a pocket involving two leucine residues of the allosteric domain and a valine and a leucine residue of the zinc domain. To probe the possible implication of this hydrophobic core in the CTP and ATP regulatory effect, the tyrosine was replaced by a phenylalanine through oligonucleotide-directed mutagenesis. Interestingly, the resulting mutant shows a complete inversion of the ATP effect; it is now inhibited by ATP instead of being activated by this nucleotide triphosphate. This mutant remains normally sensitive to the feedback inhibitor CTP. This result shows that the hydrophobic interface between the two domains of the regulatory chain plays an important role in the discrimination between the regulatory signals promoted by the two allosteric effectors.**

Escherichia coli aspartate transcarbamoylase (ATCase; aspartate carbamoyltransferase, carbamoyl-phosphate:L-aspartate carbamoyltransferase, EC 2.1.3.2) catalyzes the first committed step of pyrimidine biosynthesis—that is, the carbamoylation of the amino group of aspartate by carbamoyl phosphate. This enzyme is extensively studied as a model for cooperativity and allostery, and its structure and properties have recently been reviewed (1–3). ATCase shows homotropic cooperative interactions between the catalytic sites for aspartate binding and possibly catalysis. These interactions are explained by a transition of the enzyme from a conformation that has a low affinity for aspartate to a conformation that has a high affinity for this substrate (4–6). The crystallographic structure of these two extreme conformations is known with a resolution of 2.4 Å (7–10). Basically, ATCase is made of two catalytic trimers (catalytic subunits), which are held together through their interaction with three regulatory dimers (regulatory subunits) on which the regulatory sites are located.

CTP, the end product of the pathway, inhibits ATCase activity. This feedback inhibition is synergistically enhanced in the presence of UTP, which, by itself, has no effect (11). On the contrary, ATP activates ATCase. This antagonism is assumed to play a role in maintaining a balance between the intracellular pools of purine and pyrimidine nucleotides. ATP and CTP bind competitively to the regulatory sites, which are located in the N-terminal domain of the regulatory chains (12). The first models that were proposed to account for the regulatory properties of ATCase postulated that the nucleotides act directly on the transition involved in the homotropic cooperative interactions between the catalytic sites for aspartate binding (13, 14). However, over the years, numerous indications have accumulated suggesting that such is not the case (for a review, see ref. 15; refs. 1 and 2), and it has been proposed that, in the presence of the substrate aspartate, the nucleotides act through the combination of a primary and a secondary effect (16, 17). The effects of the nucleotides are best explained by local conformational changes, which either alter the affinity of the catalytic sites for aspartate (primary–secondary effect mechanism) (16, 17) or modulate the stability of the interfaces between regulatory and catalytic chains, thus facilitating (ATP) or hindering (CTP) the transition from T to R state upon substrate binding (effector-modulated transition) (18). X-ray solution scattering experiments confirmed the existence of such a mechanism (19). Using equilibrium isotope exchange kinetics, Hsuanyu and Wedler (15) came to the same conclusion—that ATP and CTP do not act directly on the T $\rightleftharpoons$ R equilibrium. These authors showed, in addition, that the two effectors alter the rate constant for the binding of aspartate to the catalytic sites.

The crystallographic structures of the T and R states ligated with either ATP or CTP have been solved to a resolution of 2.6 and 2.8 Å, respectively (20, 21). The ATP-ligated ATCase is in the T state. None of the hydrogen bonds or other polar interactions characteristic of this state are disrupted upon ATP binding to the unliganded enzyme, confirming that ATP does not promote the transition from T to R. Upon ATP binding, however, the distance between the two catalytic trimers increases by 0.4 Å, a change that is very small when compared to the 11 Å increase in that distance that occurs during the T to R transition. This small movement can still be considered as part of the primary effect, which, by definition, has no direct influence on the T/R proportion.

The properties of mutants, altered in the region of contact between the catalytic and the regulatory chains, suggest that the primary effects of ATP and CTP involve different interfaces between these chains (18). In fact, the ATP signal seems to be propagated mainly through the R1C1 interface (between a regulatory chain and a catalytic chain that belong to the same half of the molecule), whereas the CTP signal is propagated to a large extent through the R1C4 interface (between a regulatory chain and a catalytic chain that belongs to the other half of the enzyme molecule) (18).

The regulatory chains are folded in two domains, the effector binding domain and the zinc binding domain, which makes the contacts with the catalytic chains. Thus both the

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Abbreviation: ATCase, aspartate transcarbamoylase.

CTP and the ATP regulatory signals must necessarily be transmitted through the zinc domain. This prompted us to look for molecular interactions that might be involved in signal transmission at the interface between the allosteric effector binding domain and the zinc domain. At this interface the side chains of several residues contribute to a hydrophobic core (7, 9, 10). Leucine-32 and leucine-76 of the allosteric domain are in contact with valine-106 and leucine-151 of the zinc domain, forming a hydrophobic pocket in which the side chain of tyrosine-77 is inserted (Fig. 1). On the basis of this crystallographic information (7, 10), our previous data (22), and structural simulations (23), tyrosine-77 was replaced by a phenylalanine by using site-directed mutagenesis. This modification leads to the complete inversion of the effect of ATP on the activity of the enzyme.

MATERIALS AND METHODS

Chemicals. Carbamoyl phosphate (lithium salt), L-aspartate, ATP (sodium salt), and CTP (sodium salt) were purchased from Sigma; Tris was obtained from Merck, and L-[U-^{14}C]aspartate (300 mCi/mmol; 1 Ci = 37 GBq) was from CEA-Saclay (Gif-sur-Yvette, France).

Enzyme Assay. The ATCase activity was measured as described (24), in the presence of 50 mM Tris·HCl (pH 8), 5 mM carbamoyl phosphate, and aspartate as indicated. The protein concentrations were determined by the method of Lowry *et al.* (25), using bovine serum albumin as a standard and taking into account the 20% overestimate that is given by this method (26). The specific activity of the different enzyme species was expressed as μmol of carbamoylaspartate formed per hr per mg of protein. The kinetic parameters were calculated by an iterative nonlinear Gauss–Newton least-squares method using computer programs developed in this laboratory by P. Tauc. The influence of ATP and CTP on the rate of reaction was determined as described (16, 27). The percentage of stimulation by ATP is expressed as follows:

$$\% = \frac{(V_n - V_0) \times 100}{V_0}, \qquad [1]$$

where V_0 is the rate of reaction in the absence of ATP and V_n is the rate of reaction in its presence. The percentage of inhibition by CTP is expressed as follows:

$$\% = \frac{(V_0 - V_n) \times 100}{V_0}, \qquad [2]$$

where V_0 is the rate of reaction in the absence of CTP and V_n is the rate of reaction in its presence.

Preparation of a Mutant Form of ATCase in Which Tyrosine-77 Was Replaced by Phenylalanine (rPhe77-ATCase). Tyrosine-77 of the regulatory chain was replaced by phenylalanine using oligonucleotide-directed mutagenesis performed according to Sayers *et al.* (28) on the *pyrBI* genes cloned into an M13 phage derivative (*pyrB* and *pyrI* code for the catalytic and regulatory chains, respectively). A full-sequence DNA cassette harboring the mutation was then transferred to a suitably deleted *pyrBI* expression vector derived from pUC18, as described by Xi *et al.* (29), and transformed into *Escherichia coli* strain EK 1104 (*ara, Δpro-lac, strA, thi, ΔpyrB, pyrF*$^{\pm}$, *rpsL*) (30) in which physiological derepression of the *pyr* genes can be achieved upon uracil deprivation.

RESULTS

Aspartate Saturation Curve of rPhe77-ATCase. The aspartate saturation curve of rPhe77-ATCase is shown in Fig. 2*A* in comparison with that of the wild-type enzyme. The mutant does not exhibit homotropic cooperative interactions between the catalytic sites, a conclusion that is substantiated by the corresponding Eadie plot (Fig. 2*B*). The calculated kinetic parameters are presented in Table 1, together with those of the catalytic subunits isolated from both rPhe77-ATCase and the wild-type enzyme. The values obtained confirm the lack of homotropic cooperative interactions in rPhe77-ATCase and show a 2-fold decrease of the maximal velocity of this enzyme. The apparent K_m of the modified enzyme is about 3 times higher than the midsaturation aspartate concentration ($S_{0.5}$) of the wild-type ATCase. These changes in kinetic parameters are indeed due to the modification of the regu-

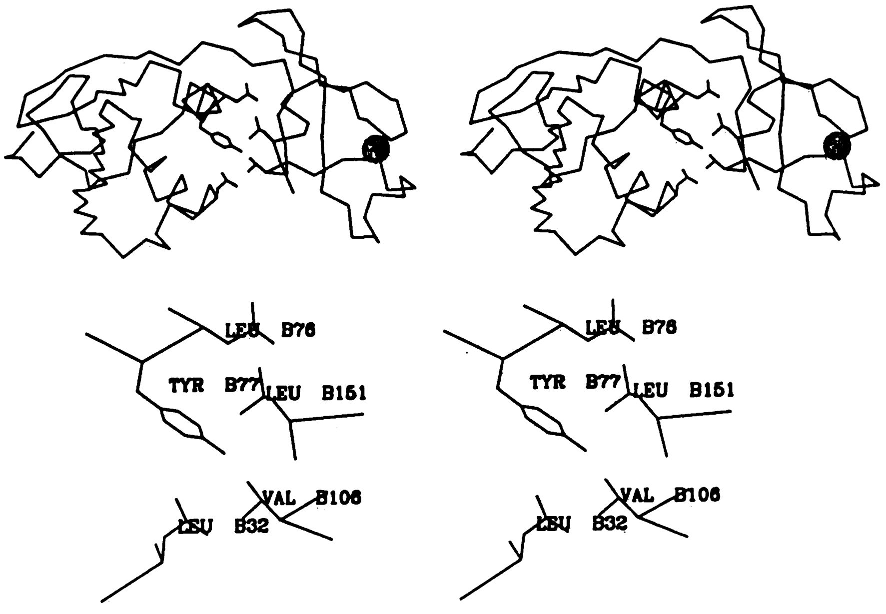

FIG. 1. Hydrophobic interface between the two domains of the regulatory chain of ATCase. (*Upper*) General stereoview of the α-carbon backbone of the two domains of the regulatory chain showing Tyr-77 and the residues that contribute to the hydrophobic pocket: Leu-32 and Leu-76 in the allosteric domain and Val-106 and Leu-151 in the zinc domain. The circle represents the zinc atom. (*Lower*) Close-up view of the hydrophobic interface between the two domains.

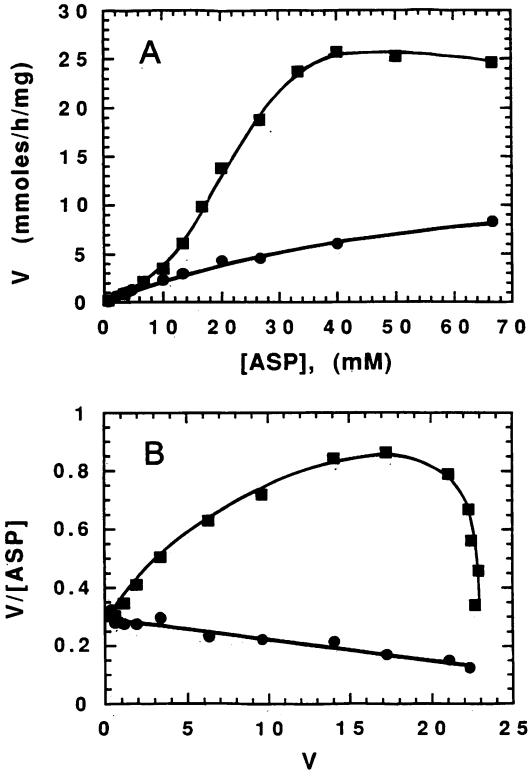

FIG. 2. Aspartate saturation curve of rPhe77-ATCase. (*A*) Saturation curves. The ATCase activity was measured as indicated in *Materials and Methods*. ■, Wild-type ATCase; ●, Phe77-ATCase. (*B*) Corresponding Eadie plots.

latory chains since, as expected, the kinetic properties of the rPhe-77 catalytic subunits are indistinguishable from those of the wild-type catalytic subunits (Table 1).

Influence of CTP on the Activity of rPhe77-ATCase. Fig. 3 shows that CTP is almost as efficient an inhibitor of the activity of rPhe77-ATCase as it is of wild-type ATCase. At 10 mM CTP, a concentration that is close to saturation, and in the presence of 5 mM aspartate, rPhe77-ATCase is inhibited by 65% as compared to 80% for the wild type. Such a difference is expected since in rPhe77-ATCase cooperativity between the catalytic sites is abolished (17).

Influence of ATP on the Activity of rPhe77-ATCase. The influence of ATP on the activity of rPhe77-ATCase was measured in the presence of 5 mM aspartate and compared to its influence on wild-type ATCase in the presence of 2 mM aspartate, thus taking into account the different affinities of the two enzymes for aspartate. The results obtained are shown in Fig. 4*A*. At that aspartate concentration, wild-type ATCase is essentially in the T state and shows the maximal 250% stimulation characteristic of this form (17). In contrast, it appears that ATP behaves as an inhibitor towards rPhe77-ATCase. A direct comparison of the effects of ATP and CTP on the activity of rPhe77-ATCase shows that ATP inhibits this enzyme just as efficiently as CTP does (Fig. 4*B*).

Table 1. Kinetic parameters of rPhe77-ATCase

Enzyme	V_m	K_m	n_H
ATCase	25,525 ± 1250	16.5 ± 1.1	2.8 ± 0.3
rPhe77-ATCase	14,300 ± 750	50.8 ± 5.2	1.0
wt catalytic subunit	32,110 ± 1960	20.8 ± 1.4	1.0
rPhe77 catalytic subunit	31,680 ± 2160	21.1 ± 2.8	1.0

These parameters were calculated as indicated in *Materials and Methods* through a computer fit to either the Hill equation or the Michaelis–Menten equation. In the case of rPhe77-ATCase, the latter gave the better correlation coefficient. V_m is expressed as μmol of carbamoylaspartate formed per hr per mg of protein, and K_m is expressed in mM. wt, Wild type; n_H, Hill coefficient.

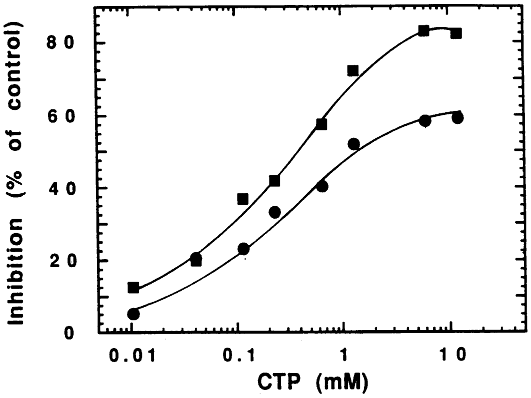

FIG. 3. Influence of CTP on the activity of Phe77-ATCase. The ATCase activity was measured; the influence of CTP on the rate of reaction was determined, and the percentage of inhibition was calculated. ■, Wild-type ATCase; ●, Phe77-ATCase.

DISCUSSION

The mutant form of ATCase in which tyrosine-77 of the regulatory chains is replaced by a phenylalanine residue does not show homotropic cooperative interactions between the catalytic sites. This enzyme is still sensitive to the effectors CTP and ATP, although its response to the latter is inverted, as discussed further. Thus this mutant provides an additional example of the previously reported uncoupling of homotropic

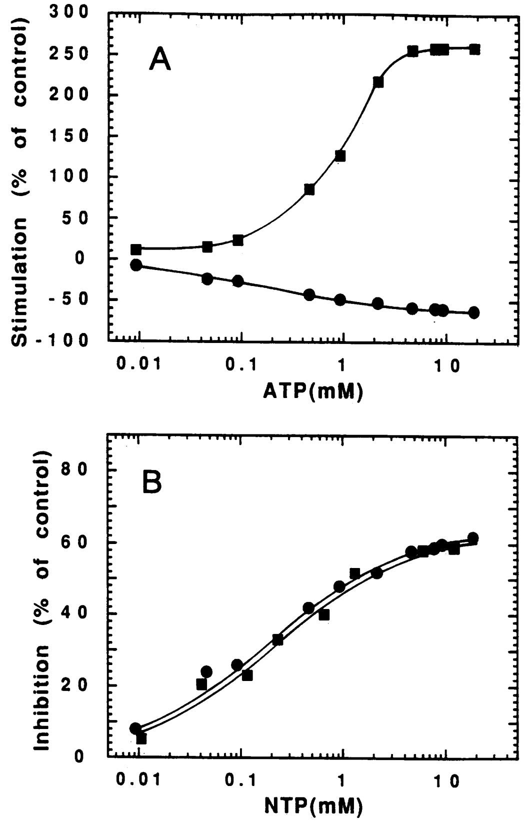

FIG. 4. Influence of ATP on the activity of Phe77-ATCase. (*A*) The influence of ATP on the rate of reaction was determined, and the percentage of stimulation was calculated as described in *Materials and Methods*. ■, Wild-type ATCase; ●, Phe77-ATCase. (*B*) Comparison of the inhibitory effect of ATP (●) and CTP (■) on the activity of rPhe77-ATCase.

and heterotropic interactions. This lack of cooperativity indicates that, together with the R1C4 (7, 10, 23, 29, 31, 32) and the C1C4 interfaces (10, 33, 34), the hydrophobic core between the two domains of the regulatory chains is another important structural feature for the establishment of the T state and consequently for the existence of homotropic cooperative interactions between the catalytic sites. The replacement of tyrosine-77 by a phenylalanine also results in a 2-fold decrease of the maximal catalytic activity of the enzyme. These two properties of rPhe77-ATCase are very similar to those of a mutant described by Eisenstein *et al.* (35) in which asparagine-111 of the regulatory chain was replaced by an alanine residue.

rPhe77-ATCase is normally sensitive to CTP, indicating that tyrosine-77 and the hydrophobic core between the two domains of the regulatory chains are not essential for transmission of the CTP signal but are important for transmission of the ATP signal, a situation that is opposite to that observed upon modification of the R1C4 interface (18). This result provides additional evidence that the mechanisms of CTP inhibition and ATP activation involve at least in part different interfaces between subunits and domains.

The most striking property of rPhe77-ATCase is the complete inversion of the ATP effect. This is an example of a modified ATCase that differently interprets the ATP signal. Modified forms of phosphofructokinase that show a reversal of the action of the effectors (36, 37) have been engineered, although in that case the modifications involve an amino acid residue located in the effector binding site. The results reported here show that the structural motif constituted by the hydrophobic interface and tyrosine-77 (Fig. 1) plays a crucial role in the discrimination between the ATP and CTP signals. The truncation of the hydroxyl group of tyrosine-77 perturbs the environment of this amino acid, possibly by promoting an increased hydrophobic packing at the interface, which might reorient the two domains of the regulatory chains. Whatever the structural consequence of the modification, it might either perturb the ATP signal at the level of the interface where the mutation is located or exert a long-range effect at the level of the effector binding site or at the interface between the catalytic and regulatory subunits.

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1. Allewell, N. M. (1989) *Annu. Rev. Biophys. Biophys. Chem.* **18**, 71–92.
2. Hervé, G. (1989) in *Allosteric Enzymes*, ed. Hervé, G. (CRC, Boca Raton, FL), pp. 61–79.
3. Kantrowitz, E. R. & Lipscomb, W. N. (1990) *Trends Biochem. Sci.* **15**, 53–59.
4. Howlett, G. J. & Schachman, H. K. (1977) *Biochemistry* **16**, 5077–5083.
5. Moody, M. F., Vachette, P. & Foote, A. M. (1979) *J. Mol. Biol.* **133**, 517–532.
6. Krause, K. L., Volz, K. W. & Lipscomb, W. N. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1643–1647.
7. Honzatko, R. B., Crawford, J. L., Monaco, H. L., Ladner, J. E., Edwards, B. F. P., Evans, D. R., Warren, S. G., Wiley, D. C., Ladner, R. C. & Lipscomb, W. N. (1982) *J. Mol. Biol.* **160**, 219–263.
8. Ke, H. M., Honzatko, R. B. & Lipscomb, W. N. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 4037–4040.
9. Krause, K. L., Volz, K. W. & Lipscomb, W. N. (1987) *J. Mol. Biol.* **193**, 527–553.
10. Ke, H., Lipscomb, W. N., Cho, Y. & Honzatko, R. B. (1988) *J. Mol. Biol.* **204**, 725–747.
11. Wild, J. R., Loughrey-Chen, S. J. & Corder, T. S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 46–50.
12. Honzatko, R. B. & Lipscomb, W. N. (1982) *J. Mol. Biol.* **160**, 265–286.
13. Changeux, J. P. & Rubin, M. M. (1968) *Biochemistry* **7**, 553–560.
14. Howlett, G. J., Blackburn, M. N., Compton, J. G. & Schachman, H. K. (1977) *Biochemistry* **16**, 5091–5100.
15. Hsuanyu, Y. & Wedler, F. (1988) *J. Biol. Chem.* **263**, 4172–4178.
16. Thiry, L. & Hervé, G. (1978) *J. Mol. Biol.* **125**, 515–534.
17. Tauc, P., Leconte, C., Kerbiriou, D., Thiry, L. & Hervé, G. (1982) *J. Mol. Biol.* **155**, 155–168.
18. Xi, X.-G., Van Vliet, F., Ladjimi, M. M., de Wannemaeker, B., De Staercke, C., Glansdorff, N., Piérard, A., Cunin, R. & Hervé, G. (1991) *J. Mol. Biol.* **220**, 789–799.
19. Hervé, G., Moody, M. F., Tauc, P., Vachette, P. & Jones, P. T. (1985) *J. Mol. Biol.* **185**, 189–199.
20. Stevens, R. C., Gouaux, J. E. & Lipscomb, W. N. (1990) *Biochemistry* **29**, 7691–7701.
21. Gouaux, J. E., Stevens, R. C. & Lipscomb, W. N. (1990) *Biochemistry* **29**, 7702–7715.
22. Ladjimi, M. M., Ghelis, C., Feller, A., Cunin, R., Glansdorff, N., Piérard, A. & Hervé, G. (1985) *J. Mol. Biol.* **186**, 715–724.
23. Cherfils, J., Vachette, P., Tauc, P. & Janin, J. (1987) *EMBO J.* **6**, 2843–2847.
24. Perbal, B. & Hervé, G. (1972) *J. Mol. Biol.* **70**, 511–529.
25. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
26. Kerbiriou, D., Hervé, G. & Griffin, J. H. (1977) *J. Biol. Chem.* **252**, 2881–2890.
27. Kerbiriou, D. & Hervé, G. (1972) *J. Mol. Biol.* **64**, 379–392.
28. Sayers, J. R., Schmidt, W. & Eckstein, F. (1988) *Nucleic Acids Res.* **16**, 791–802.
29. Xi, X.-G., Van Vliet, F., Ladjimi, M., Cunin, R. & Hervé, G. (1990) *Biochemistry* **29**, 8491–8498.
30. Nowlan, S. F. & Kantrowitz, E. R. (1985) *J. Biol. Chem.* **260**, 14712–14716.
31. Cunin, R., Van Vliet, F., Glansdorff, N., Xi, X.-G., Hervé, G., Ladjimi, M. M., Stalon, V. & Piérard, A. (1989) *Arch. Int. Physiol. Biochim.* **97**, B135.
32. Newton, C. J. & Kantrowitz, E. R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2309–2313.
33. Ladjimi, M. M. & Kantrowitz, E. R. (1988) *Biochemistry* **27**, 276–283.
34. Middleton, S. A. & Kantrowitz, E. R. (1988) *Biochemistry* **27**, 8653–8660.
35. Eisenstein, E., Markby, D. W. & Schachman, H. K. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 394–398.
36. Lau, F. T. & Fersht, A. (1987) *Nature (London)* **326**, 811–812.
37. Lau, F. T. & Fersht, A. (1989) *Biochemistry* **28**, 6841–6847.