Ligand-mediated conformational changes in wheat-germ aspartate transcarbamoylase indicated by proteolytic susceptibility

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Ligand-mediated effects on the inactivation of pure wheat-germ aspartate transcarbamoylase by trypsin were examined. Inactivation was apparently first-order in all cases, and the effects of ligand concentration on the pseudo-first-order rate constant, k , were studied. Increase in k (labilization) was effected by carbamoyl phosphate, phosphate and the putative transition-state analogue, *N*-phosphonoacetyl-L-aspartate. Decrease in k (protection) was effected by the end-product inhibitor, UMP, and by the ligand pairs aspartate/phosphate and succinate/carbamoyl phosphate, but not by aspartate or succinate alone up to 10 mM. Except for protection by the latter ligand pairs, all other ligand-mediated effects were also observed on inactivation of the enzyme by Pronase and chymotrypsin. Ligand-mediated effects on the fragmentation of the polypeptide chain by trypsin were examined electrophoretically. Slight labilization of the chain was observed in the presence of carbamoyl phosphate, phosphate and *N*-phosphonoacetyl-L-aspartate. An extensive protection by UMP was observed, which apparently included all trypsin-sensitive peptide bonds. No significant effect by the ligand pair succinate/carbamoyl phosphate was noted. It is concluded from these observations that UMP triggers an extensive, probably co-operative, transition to a proteinase-resistant conformation, and that carbamoyl phosphate similarly triggers a transition to an alternative, proteinase-sensitive, conformation. These antagonistic conformational changes may account for the regulatory kinetic effects reported elsewhere [Yon (1984) *Biochem. J.* 221, 281–287]. The protective effect by the ligand pairs aspartate/phosphate and succinate/carbamoyl phosphate, which operates only against trypsin, is concluded to be due to local shielding of essential lysine or arginine residues in the aspartate-binding pocket of the active site, to which aspartate (or its analogue, succinate) can only bind as part of a ternary complex.

Aspartate transcarbamoylase (L-aspartate: carbamoyl phosphate carbamoyltransferase, EC 2.1.3.2) has been purified to homogeneity from wheat germ (Yon, 1981). The enzyme catalyses a compulsory-order bisubstrate reaction with carbamoyl phosphate as the leading substrate (Grayson *et al.*, 1979). Like several bacterial forms of the enzyme, it is a comparatively small trimer of molecular mass 104 kDa (Vickers, 1981; Yon *et al.*, 1982). Unlike these non-co-operative bacterial enzymes, however, it shows a highly co-operative regulatory mechanism. The regulatory kinetics of the relatively crude enzyme (Yon, 1972) and more recently of the highly purified enzyme (Yon, 1984) have been examined. Half-saturation concen-

trations of the leading substrate, carbamoyl phosphate, and of the end-product inhibitor, UMP, were found to be interdependent, and this interdependence was tentatively interpreted in terms of a modified concerted-transition conformational mechanism, in which alternative conformational states, each binding one of the two ligands preferentially, were postulated (Monod *et al.*, 1965).

Ligand-mediated changes in susceptibility to proteolysis have been cited as evidence of conformational change in several cases (reviewed by Citri, 1973). Such evidence is especially compelling when local steric-hindrance effects by the ligand can be ruled out as the major contributing factor,

as, for example, when proteinases of differing specificity show parallel effects, or when the effect of the ligand can be shown to extend to many more than one or two sensitive peptide bonds. In a previous study (Yon, 1973) the effects of several inactivating agents, including trypsin, on the activity of aspartate transcarbamoylase in a crude extract of wheat germ were examined. Only qualitative conclusions could be drawn, however, on account of the complex nature of the crude protein sample. In the present work we have used a highly purified preparation of the enzyme, and studied the effects of ligands on a quantifiable parameter, the pseudo-first-order rate constant for proteolytic inactivation. We have also examined electrophoretically the effects of ligands on the tryptic fragmentation of the polypeptide chain. We conclude that, with one exception, these effects may be attributed to conformational changes in the enzyme protein. In particular, such conformational changes provide a possible mechanism for the regulatory kinetic effects reported in the preceding paper (Yon, 1984).

Experimental

Materials

Aspartate transcarbamoylase was purified from wheat germ as described by Yon (1981), with the exception that the two organic-solvent fractionation steps were combined. The enzyme was shown to be more than 95% pure by polyacrylamide-gel electrophoresis. Trypsin, chymotrypsin and Pronase (a wide-specificity proteinase from *Streptomyces griseus*) were obtained from Sigma Chemical Co., and were used without further purification. Enzyme substrates, other ligands and buffer components, were from Sigma Chemical Co. or BDH Chemicals. *N*-Phosphonoacetyl-L-aspartate was synthesized by the procedure of Goodson *et al.* (1980) and shown to be approx. 90% pure by aspartate analysis (Locarte amino acid auto-analyser) after 24 h hydrolysis in 6M-HCl at 105°C.

By using the hydrolysis of casein (Laskowski, 1955) as test protein, 1,6-diaminohexane (5mM) was found to inhibit trypsin completely, but to have no effect on chymotrypsin (both at 0.025%, w/v). Similarly, the activity of chymotrypsin was completely inhibited by 2-nitro-4-carboxyphenyl *NN'*-diphenylcarbamate, which had no detectable effect on trypsin. These results were taken to indicate that the trypsin and chymotrypsin preparations were each essentially uncontaminated by the other.

Proteolytic inactivation of aspartate transcarbamoylase

All solutions were pre-equilibrated to 25°C and the reactions were carried out at this temperature.

In a small tube were mixed 1.88 ml of 0.1M-Tris/acetic acid buffer, pH 8.5, containing the appropriate ligand, and 0.02 ml of purified aspartate transcarbamoylase (approx. 20 µg) stored as recommended by Yon (1981). Proteolysis was started by adding 0.10 ml of trypsin in the same buffer, to give a final concentration of 0.0025% (w/v). Zero-time samples were removed for assay immediately, and thereafter duplicate samples (0.1 ml) were withdrawn at 5 min intervals. These samples were transferred to assay tubes containing 1.2 ml of 1.25mM-L-aspartate in 0.1M-glycine/NaOH, pH 10, at 25°C, containing 0.5% (w/v) *p*-aminobenzamidine to inhibit further tryptic activity. When all timed samples had been collected (20 min in most cases) aspartate transcarbamoylase activity was initiated by adding 0.1 ml of aqueous 15mM-carbamoyl phosphate to each assay tube. The reaction was terminated after 20 min by the addition of 1.0 ml of reagent 1 of Prescott & Jones (1969). Enzymically produced carbamoylaspartate was determined colorimetrically as described by these same authors, under rigorously controlled room lighting.

Proteolysis by chymotrypsin (final concn. 0.025%, w/v) and by Pronase (0.0025%, w/v) were performed under the same conditions as described for trypsin, except that for chymotrypsin inhibition in the assay tubes 2-nitro-4-carboxyphenyl *NN'*-diphenylcarbamate (0.5%, w/v) was used. Trial experiments were performed to show that in the presence of these inhibitors there was negligible further proteolysis in the assay tubes, and that the inhibitors had no effects on aspartate transcarbamoylase activity or on the colorimetric assay of carbamoylaspartate. Experiments were also performed to show that ligands of aspartate transcarbamoylase had no effect on tryptic hydrolysis of casein (Laskowski, 1955).

Fragmentation of polypeptide chains of aspartate transcarbamoylase by trypsin

Fragmentation of these chains was investigated in reaction mixtures containing a 16-fold excess by weight of aspartate transcarbamoylase over trypsin. Small tubes containing 250 µg of aspartate transcarbamoylase were incubated with 15 µg of trypsin at 25°C in a total volume of 0.5 ml. The buffer contained 0.1M-Tris/acetic acid, pH 8.5, and ligands at various concentrations. Samples (60 µl) were withdrawn at 30 min intervals and added to a 5-fold molar excess of *p*-aminobenzamidine over trypsin to stop proteolysis. Samples were then denatured, together with non-digested controls, and subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the procedure of Weber & Osborn (1969).

Results

Ligand-mediated effects on inactivation by trypsin

(a) *Effect of UMP.* Fig. 1(a) shows apparent first-order plots of the inactivation of aspartate transcarbamoylase by trypsin. Pseudo-first-order rate constants, k , obtained from the slopes of these plots, are plotted against the UMP concentration in Fig. 1(b). UMP is seen to have a protective effect

Fig. 1. Effect of UMP on the tryptic inactivation of wheat-germ aspartate transcarbamoylase

(a) First-order plots of inactivation in the absence of UMP (●) and in the presence of UMP at concentrations (μM) of 1 (○), 2 (▲), 3 (△), 5 (■), 7 (□), 8 (▼), 10 (▽), 15 (◆) and 20 (◇). (b) Pseudo-first-order rate constants from (a) replotted versus UMP concentration.

on enzyme activity, k decreasing from 0.056 min⁻¹ in the absence of UMP to 0.003 min⁻¹ in saturating UMP. The protection is not complete, however, since a finite inactivation rate persists on saturation with UMP. The rate constant is also seen to be sigmoidally dependent on UMP concentration. Sigmoidicity and saturability suggest strongly that the protective effect is transmitted co-operatively between subunits of the enzyme, on binding UMP. By using the analogous double-logarithmic (Hill) plot (Citri, 1973), the half-saturation concentration of UMP was found to be 3.8 μM and the co-operativity (Hill) coefficient to be 3.2. Since there is strong evidence that the enzyme is a trimer (Yon *et al.*, 1982), it is logical to assume three UMP-binding sites, one per subunit. Coincidence of the Hill coefficient and the number of interacting sites indicates a high degree of co-operativity, possibly approaching a concerted effect.

(b) *Effect of carbamoyl phosphate.* In contrast with UMP, carbamoyl phosphate labilizes the enzyme. Inactivation is again first-order (Fig. 2a), and the relation between pseudo-first-order rate constant, k , and carbamoyl phosphate concentration is given in Fig. 2(b). The effect of carbamoyl phosphate, like that of UMP, is sigmoidal and saturable, suggesting a co-operative transition to a trypsin-sensitive state. Saturation with carbamoyl phosphate increases the inactivation rate constant from 0.054 to 0.177 min⁻¹. Analysis by a Hill plot (Citri, 1973) indicates a half-saturating concentration of carbamoyl phosphate of 20 μM, and a Hill coefficient of 3.1. Hence the comments on co-operativity, made above with respect to UMP, also apply to carbamoyl phosphate.

(c) *Combined effects of UMP and carbamoyl phosphate.* Addition of gradual increments of UMP to an enzyme solution saturated with respect to labilization by carbamoyl phosphate resulted in a smooth transition from an inactivation rate constant of 0.180 min⁻¹ to one of 0.003 min⁻¹. The converse transition was observed when increments of carbamoyl phosphate were added to an enzyme solution saturated with respect to protection by UMP. These results confirm that the effects seen in (a) and (b) above are fully reversible, and occur in the combined presence of both ligands.

(d) *Effect of phosphate.* Phosphate is a product of the reaction catalysed by aspartate transcarbamoylase, and has been shown to be competitive with carbamoyl phosphate in an ordered bisubstrate reaction (Grayson *et al.*, 1979). Fig. 3(a) shows that phosphate, like carbamoyl phosphate, labilizes the enzyme with respect to inactivation by trypsin, the inactivation rate constant, k , being related to phosphate concentration in a sigmoidal and saturable manner. However, the rate constant

Fig. 2. Effect of carbamoyl phosphate on the tryptic inactivation of wheat-germ aspartate transcarbamoylase (a) First-order plots of inactivation in the absence of carbamoyl phosphate (●) and in the presence of carbamoyl phosphate at concentrations (μM) of 10 (○), 20 (▲), 30 (△), 40 (▽), 50 (■), 75 (□) and 100 (□). (b) Pseudo-first-order rate constants from (a) replotted versus carbamoyl phosphate concentration.

at saturation (0.115 min^{-1}), the half-saturating concentration of phosphate ($280 \mu\text{M}$) and the Hill coefficient (2.1) are all different from those of carbamoyl phosphate.

(e) Effect of *N*-phosphonoacetyl-L-aspartate. This compound is a putative transition-state analogue of the reaction catalysed by aspartate trans-

Fig. 3. Effects of (a) phosphate and (b) *N*-phosphonoacetyl-L-aspartate on the tryptic inactivation of wheat-germ aspartate transcarbamoylase

The diagrams show plots of pseudo-first-order rate constants versus the concentration of phosphate or *N*-phosphonoacetyl-L-aspartate.

carbamoylase, and incorporates features of both substrates. It is kinetically competitive with carbamoyl phosphate, with an inhibition constant in the order of 10 nM in the case of the wheat-germ enzyme (Yon, 1984). Fig. 3(b) shows the effect of this compound on the inactivation rate constant. Like carbamoyl phosphate and phosphate, this compound labilizes the enzyme; however, in this case the effect appears to be hyperbolic (note that the abscissa is logarithmic in Fig. 3b). The saturating rate constant is 0.150 min^{-1} and the half-saturating concentration of *N*-phosphonoacetyl-L-aspartate is about 2 nM.

(f) Effects of L-aspartate and succinate. L-Aspartate had no detectable effect on the inactivation rate at concentrations up to and including 10 mM (about 100 times greater than its Michaelis constant). A slight protection was observed at 20 mM (decrease in k from 0.054 to 0.041 min^{-1}). The effect of succinate was essentially identical.

(g) *Effect of aspartate in combination with phosphate.* Unlike its effect when alone, aspartate in the presence of phosphate showed a marked protective effect, being able to reverse the labilizing effect of phosphate (Fig. 4a). The dependence of k on aspartate concentration was hyperbolic, the value of k at saturation being 0.025 min^{-1} . The half-saturation concentration of aspartate was $150 \mu\text{M}$, essentially the same as its Michaelis constant of $120 \mu\text{M}$ (Grayson *et al.*, 1979). The dependence of the aspartate effect on the presence of phosphate is ascribed to ordered ligand-binding in which aspartate can only bind to the binary enzyme-phosphate complex (Grayson *et al.*, 1979).

(h) *Effect of succinate in combination with carbamoyl phosphate.* In a very similar way to aspartate (preceding paragraph), succinate showed a protective effect when in the presence of carbamoyl phosphate (Fig. 4b). Quantitative aspects are essentially identical with those in the preceding paragraph; in particular, the half-saturating concentration of succinate ($140 \mu\text{M}$) and its inhibition constant ($210 \mu\text{M}$) (Grayson *et al.*, 1979) are of similar order. The comments on ordered binding apply here also.

Comparison of ligand effects on inactivation by trypsin, chymotrypsin and Pronase

Each ligand was tested at a single concentration (see Fig. 5 legend) on inactivation by each of the proteolytic enzymes. The inactivation followed apparent first-order kinetics in every case, and the relative values of the rate constants, k , are presented as a histogram in Fig. 5. The proteinase concentrations were 0.0025% (w/v) for trypsin and Pronase and 0.025% (w/v) for chymotrypsin, to give roughly similar inactivation rates in the absence of ligands. Fig. 5 shows that, with two exceptions, ligands or ligand combinations have remarkably similar effects on inactivation by all three proteinases, both qualitatively and quantitatively. Thus in all cases UMP is protective, the same concentration of UMP producing nearly the same degree of protection in each case (Figs. 5b and 5c). Again, in all cases carbamoyl phosphate labilizes the enzyme, similar increases in k being produced by a given concentration of the substrate. The same comments may be made for the effects of phosphate (Fig. 5f) and *N*-phosphonoacetyl-L-aspartate (Fig. 5h). The exceptions are the effects of the paired ligands aspartate/phosphate and succinate/carbamoyl phosphate (Figs. 5e and 5g), where the protection (decrease in k relative to that of phosphate or carbamoyl phosphate alone) is seen to be specific to inactivation by trypsin.

Fragmentation of the polypeptide chain by trypsin

It has been shown that wheat-germ aspartate transcarbamoylase contains three similar or identical polypeptide chains of mass approx. 37 kDa (Yon *et al.*, 1982). The fragmentation of these chains in the presence of trypsin and various ligands was observed at 30 min intervals for 3 h by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The fragmentation patterns, with-out and with various ligands, are shown in Figs. 6(a) and 6(b) after digestion for 1 h and 3 h respectively; other intermediate times gave intermediate patterns. A control sample (gel 1 in each case) shows the undigested enzyme at the same loading as in digested samples; a trace of inherent impurity is seen as a consequence of the heavy

Fig. 4. Effects of (a) aspartate plus phosphate and of (b) succinate plus carbamoyl phosphate on the tryptic inactivation of wheat-germ aspartate transcarbamoylase

(a) Pseudo-first-order rate constants for inactivation in the presence of 0.1 (Δ), 0.3 ($\bullet$) and 1.0 ($\circ$) mM-phosphate, each plotted versus aspartate concentration. (b) Pseudo-first-order rate constants for inactivation in the presence of 10 (Δ), 25 ($\bullet$) and 100 ($\circ$) μM -carbamoyl phosphate, each plotted versus succinate concentration.

Fig. 5. Comparison of the effects of ligands on the inactivation of wheat-germ aspartate transcarbamoylase by three different proteinases

The proteinases are denoted as follows: ■, trypsin; □, chymotrypsin; ▨, Pronase. In each case the pseudo-first-order rate constant for inactivation is expressed as a percentage of the rate constant for tryptic inactivation in the absence of ligands. Ligands and their concentrations were as follows: (a) none; (b) 4 μ M-UMP; (c) 20 μ M-UMP; (d) 25 μ M-carbamoyl phosphate; (e) 25 μ M-carbamoyl phosphate + 150 μ M-succinate; (f) 0.3 mM-phosphate; (g) 0.3 mM-phosphate + 140 μ M-aspartate; (h) 2 mM-N-phosphonoacetyl-L-aspartate.

loading used to show up weak bands in protein digests, which does not seriously complicate interpretation of the main fragmentation. In the presence of trypsin, various amounts of residual native chains, several large (25–33 kDa) intermediate fragments (showing as well-defined bands) and an indeterminate number of smaller fragments (showing as blurred bands in the 12–22 kDa region) are seen. The transfer of staining from higher- to lower-molecular mass species is indicative of the fragmentation rate. Compared

Fig. 6. Polyacrylamide-gel electrophoresis of wheat-germ aspartate transcarbamoylase digested by trypsin for (a) 1 h and (b) 3 h

In both cases gel 1 is a control sample of undigested enzyme. The other gels show equivalent samples of the enzyme digested in the presence of ligands as follows: gel 2, none; gel 3, 5 μ M-UMP; gel 4, 25 μ M-carbamoyl phosphate; gel 5, 25 μ M-carbamoyl phosphate + 150 μ M-succinate; gel 6, 2 mM-N-phosphonoacetyl-L-aspartate. The molecular-mass scale was established by co-electrophoresis of standard proteins. Electrophoresis was in the presence of sodium dodecyl sulphate as described by Weber & Osborn (1969).

with the absence of ligands (gel 2), carbamoyl phosphate alone, carbamoyl phosphate + succinate and N-phosphonoacetyl-L-aspartate (gels 4, 5 and 6 respectively) all appear to increase slightly

the fragmentation rate (judged mainly by increased staining density in the low-molecular-mass region). In contrast, UMP has a strongly protective effect (gel 3). An important observation is that UMP exerts some degree of protection over all, or nearly all, trypsin-sensitive bonds in the chain, since only in the presence of UMP is there clear evidence of residual intact chains even after 3 h in the presence of trypsin. The combination of carbamoyl phosphate and succinate did not produce an effect significantly different from that of carbamoyl phosphate alone.

Discussion

Several authors have used and discussed proteolytic susceptibility as a probe of protein conformation (reviewed extensively by Citri, 1973). Among these McLintock & Markus (1968) identified several conformational states of the *Escherichia coli* aspartate transcarbamoylase, which were used to propose a possible regulatory model (Markus *et al.*, 1971). Yon (1973) used tryptic hydrolysis, together with other inactivating processes, to study the effects of ligands on aspartate transcarbamoylase from wheat germ; however, only general, qualitative, conclusions were possible by reason of the relatively crude enzyme preparation that was used.

Cases of ligand-mediated labilization towards proteolysis may be attributed to conformational change with little hesitation, since it is difficult to envisage other mechanisms whereby binding of a ligand can cause increased exposure of sensitive peptide bonds (Citri, 1973). In the present study, therefore, we postulate that the binding of carbamoyl phosphate, phosphate and *N*-phosphonoacetyl-L-aspartate converts the enzyme into conformational states with decreased resistance to proteolysis. We propose further that the conformational change extends well beyond the immediate locality of the site that binds carbamoyl phosphate and its competitors, phosphate and *N*-phosphonoacetyl-L-aspartate, since (a) the inactivation of the enzyme by trypsin correlates with slight general increases in chain fragmentation involving, presumably, many trypsin-sensitive peptide bonds, (b) similar effects are seen with proteinases of widely different specificities, and (c) the sigmoidal effect of carbamoyl phosphate, in particular, on inactivation rate constant (k) suggests a co-operative effect that is transmitted between binding sites, i.e. presumably between subunits. We assume the conformations taken up in the presence of carbamoyl phosphate and its two competitors to be similar, although they are clearly non-identical since saturation with each ligand leads to a different value of k , and the effect of *N*-

phosphonoacetyl-L-aspartate is not transmitted sigmoidally.

In contrast with labilization, ligand-induced protective effects cannot be unambiguously attributed to conformational changes, since they may be due simply to steric shielding of sensitive peptide bonds located within the ligand-binding site. Two cases of ligand-induced protection were observed in these studies, one by UMP and the other by the ligand pairs aspartate/phosphate and succinate/carbamoyl phosphate. Although we cannot rule out a contribution from a local shielding effect by UMP, the protection by this ligand, we believe, derives primarily from an extensive conformational change. The evidence for this conclusion may be summarized as follows. (a) Protection against inactivation by trypsin correlates with clear evidence of protection against fragmentation of the chain; moreover, this protection appears to affect nearly every trypsin-sensitive bond in the chain (see the Results section). Preliminary amino acid analysis (S. C. J. Cole & R. J. Yon, unpublished work) indicates that lysine and arginine (the target sites for trypsin) comprise 40–50 of the residues in an estimated 330-residue chain. A substantial number of these highly polar residues must be accessible at the enzyme's surface. (b) UMP provides the same degree of protection against three proteinases of very different specificity. (c) The sigmoidal dependence of the inactivation rate constant, k , on UMP concentration argues for a co-operative transmission of the protection between UMP-binding sites, i.e. presumably between subunits. (d) It has previously been shown (Yon, 1973) that UMP provides protection against very different inactivating agents, including heating at 60°C, exposure to alkaline conditions (pH 11.3) and exposure to the detergent sodium dodecyl sulphate.

The other protective effects, by aspartate in combination with phosphate and by succinate in combination with carbamoyl phosphate, are more likely to be due to local shielding effects, since (a) they are only observed in inactivation by trypsin, and not in inactivation by chymotrypsin or Pronase, and (b) they are not correlated with more widespread changes in chain fragmentation, as the fragmentation pattern in the presence of carbamoyl phosphate alone appears to be little affected by the inclusion of succinate (Fig. 6). We conclude, from the known specificity of trypsin, that specific functional lysine and/or arginine residues in the aspartate-binding pocket of the active site are shielded by the binding (presumably electrostatically) of L-aspartate or its analogue, succinate. The carboxy groups of these ligands have been shown to be absolutely essential for binding to the enzyme (Grayson *et al.*, 1979). The dependence of

the protective effect of each of these ligands on the binding of another ligand (phosphate/carbamoyl phosphate) is entirely consistent with an ordered-binding mechanism in which the binary enzyme-carbamoyl phosphate or enzyme-phosphate complex, and not the free enzyme, is capable of binding the appropriate dicarboxylic acid (Grayson *et al.*, 1979). One observation that is not consistent with this theory, however, is the failure of the putative transition-state analogue, *N*-phosphonoacetyl-L-aspartate, to produce the same specific protection against trypsin that the postulated ternary complexes do. At present we cannot account for this discrepancy.

The apparently extensive and different conformational changes that accompany the binding of carbamoyl phosphate and UMP, and the fact that they appear to be readily reversible in the combined presence of both ligands and to depend on their relative concentrations, provide support for a conformational model of the regulation of this enzyme. A minimal model based on a concerted two-conformation mechanism (Monod *et al.*, 1965), and extended to account for certain kinetic anomalies, is proposed in the preceding paper (Yon, 1984). There are, however, a number of discrepancies between quantitative aspects of that model and of the present proteolytic-inactivation studies. Thus the concerted-transition model predicts that the free enzyme is predominantly in the conformation that exclusively binds UMP, i.e. the allosteric constant *L* is approx. 8 (see Yon, 1984). A consequence of this prediction is that the binding of UMP to the free enzyme should be hyperbolic, which conflicts with the strong co-operativity shown in Fig. 1(b) of the present paper. Moreover, there is a relatively poor correlation between the fraction of protein in a given conformational state calculated on the basis of the concerted model, on the one hand, and of the inactivation rate constant *k*, on the other. The concerted model proposed by Yon (1984) must therefore be seen as providing only an approximation to the true behaviour of the enzyme. At the cost of a considerable increase in

complexity, additional (including hybrid) conformational states might be invoked to account for the co-operativity shown by both UMP and carbamoyl phosphate binding in the present study; neither the present results nor those reported by Yon (1984) rule out alternative models such as those proposed by Koshland *et al.* (1966), for example. Further work is required, however, to first test more simple explanations, e.g. possible differences in the allosteric constant *L* between different batches of the purified enzyme.

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