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Regulatory interactions in *Arabidopsis thaliana* acetohydroxyacid synthase

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Abstract Acetohydroxyacid synthase (AHAS; EC 4.1.3.18) contains catalytic and regulatory subunits, the latter being required for sensitivity to feedback regulation by leucine, valine and isoleucine. The regulatory subunit of *Arabidopsis thaliana* AHAS possesses a sequence repeat and we have suggested previously that one repeat binds leucine while the second binds valine or isoleucine, with synergy between the two sites. We have mutated four residues in each repeat, based on a model of the regulatory subunit. The data confirm that there are separate leucine and valine/isoleucine sites, and suggest a complex pathway for regulatory signal transmission to the catalytic subunit. **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Acetohydroxyacid synthase; Branched-chain amino acid; Enzyme inhibition; Enzyme regulation; Subunit

1. Introduction

The first few steps in the biosynthesis of leucine, valine and isoleucine follow a common pathway in which the first reaction is catalysed by acetohydroxyacid synthase (AHAS; EC 4.1.3.18) [1,2]. This enzyme catalyses the decarboxylation of pyruvate followed by reaction with either pyruvate or 2-ketobutyrate to form 2-acetolactate or 2-aceto-2-hydroxybutyrate, respectively.

Bacterial AHAS is composed of two large subunits that contain the catalytic machinery, and two smaller regulatory subunits. The regulatory subunit is required for full catalytic activity and for sensitivity to feedback inhibition by the branched-chain amino acids. In agreement with earlier predictions [3] a similar subunit arrangement has now been established in eukaryotic AHAS, first in yeast [4] and later in plants [5,6].

An unusual feature of plant AHAS is that it is inhibited by all three of the branched-chain amino acids, unlike most bacterial and fungal enzymes that are sensitive to valine only. Moreover, there is synergistic inhibition by the combination of leucine plus valine. These properties are observed for the plant enzyme extracted from its native source [7] as well as that reconstituted in vitro using the purified *Arabidopsis thaliana* subunits expressed in *Escherichia coli* [6]. The synergistic inhibition has been linked to the observation that the *A. thaliana* AHAS regulatory subunit amino acid sequence shows clear evidence of an internal duplication. We have suggested that the protein contains two domains with one binding valine (or isoleucine) and the other leucine [6]. The evidence for a leucine-specific site is that reconstitution of the catalytic subunit with the one domain of the regulatory subunit yields an enzyme that is inhibited by leucine but not by valine or isoleucine. The second domain cannot bind leucine because saturation with leucine should abolish the effects of valine (or isoleucine) whereas it is observed that saturation with leucine enhances the affinity for valine. A molecular model of the *A. thaliana* AHAS regulatory subunit was proposed based on the regulatory domain of threonine deaminase (EC 4.2.1.16), which also contains an internal duplication that creates separate binding sites for its regulators valine and isoleucine [8].

In this paper we have tested this model by mutating four of the residues in each of the repeats of the *A. thaliana* AHAS regulatory subunit. The data show that each mutation produces similar effects on inhibition by valine and isoleucine while the effects on leucine inhibition are not correlated. These results support the proposal that one site binds valine or isoleucine and the other binds leucine. However, it appears that the two regulator sites are not solely contained within one repeat but may be located at the interface between two domains. This finding is in agreement with a proposed model for the bacterial enzyme [9] in which the interface involves a regulatory subunit dimer. The plant regulatory subunit displays an inherent asymmetry as judged by the observation that mutation of the equivalent residue in each repeat does not result in reciprocal effects on the inhibition. The data suggest a complex pathway for transmission of inhibitory signals within the regulatory subunit and across to the catalytic subunit.

2. Materials and methods

Materials and experimental procedures were as described previously [6], except as noted below. Methods for bacterial culture and DNA manipulation generally followed standard procedures [10]. Mutations were introduced by PCR using the megaprimer method [11]. Oligonucleotides were designed to introduce or remove a restriction enzyme recognition site to aid identification of mutated DNA. All constructs were tested by diagnostic restriction enzyme digestion and confirmed by DNA sequencing using the Prism Ready Dye Terminator Cycle Sequencing kit and DNA Sequencer 373A (Perkin-Elmer Applied Biosystems, Norwalk, CT, USA).

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Abbreviations: AHAS, acetohydroxyacid synthase; K_i^{app}, apparent inhibition constant

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Enzyme inhibition data were analysed by fitting to them the equation:

$$v = v_o((1-p/100) + (p/100)/(1 + [I]/K_i^{app}))$$

where v is the measured rate, [I] is the inhibitor concentration, v_o is the rate in the absence of inhibitors, p is the percent inhibition at saturating inhibitor concentration and K_i^{app} is the apparent inhibition constant. Fitting was performed using the GraFit program (Erithacus Software Ltd., Horley, UK) to obtain best estimates and standard errors for the parameters v_o, p and K_i^{app}.

3. Results and discussion

Comparison of various known or presumed AHAS regulatory subunit sequences has identified a region that shows a high degree of conservation (Fig. 1A). A glycine residue (G98 and G331 in the first and second repeats, respectively, of the *A. thaliana* protein) is totally conserved except in *E. coli* AHAS isoenzyme II where it is a glutamate. This *E. coli* AHAS isoform is valine-insensitive and it has been shown that mutation of the corresponding glycine to aspartate in *E. coli* AHAS isoenzyme III [9] and *Streptomyces cinnamonensis* AHAS [12] abolishes valine inhibition. Another residue that is invariant in all of the proteins except *E. coli* AHAS isoenzyme II is N113/N346 and this residue has been mutated to histidine in *E. coli* AHAS isoenzyme III [9] resulting in a loss of valine sensitivity.

In our molecular model of the *A. thaliana* AHAS regulatory subunit (Fig. 1B) G98 is close to N346 in the other repeat and, similarly, G331 is close to N113. Two residues flanking these in space are F107/F340 and Y112/Y345. While these residues are not totally conserved in AHAS regulatory subunit sequences, they are each invariably aromatic or large hydrophobic residues.

These observations led us to perform mutation studies on each of the residues shown in Fig. 1B. Apart from the glycine residues, each was mutated to alanine and N346 in repeat 2 was also mutated to histidine, as found in the valine-insensitive *E. coli* AHAS isoenzyme II. G98 was changed to a glu-

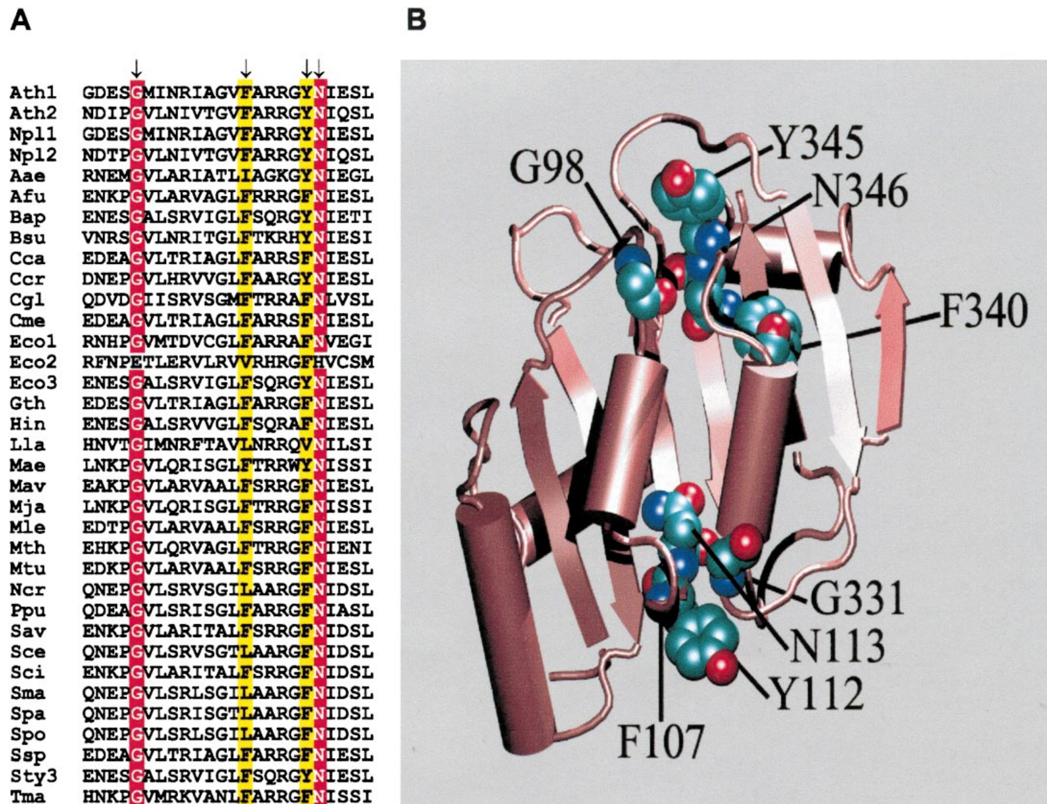


Fig. 1. Structure of the AHAS regulatory subunit. Panel A shows an alignment of the highly conserved section of the known and presumed AHAS regulatory subunit protein sequences. The plant (Ath1 and Ath2, *A. thaliana* repeats 1 and 2; Npl1 and Npl2, *Nicotiana plumbaginifolia* repeats 1 and 2) are shown first. Other sequences are arranged in alphabetical order by species abbreviation. These abbreviations are: Aae, *Aquifex aeolicus*; Afu, *Archaeoglobus fulgidus*; Bap, *Buchnera aphidicola*; Bsu, *Bacillus subtilis*; Cca, *Cyanidium caldarium*; Ccr, *Caulobacter crescentus*; Cgl, *Corynebacterium glutamicum*; Cme, *Cyanidioschyzon merolae*; Eco1, *E. coli* isozyme I; Eco2, *E. coli* isozyme II; Eco3, *E. coli* isozyme III; Gth, *Guillardia theta*; Hin, *Haemophilus influenzae*; Lla, *Lactococcus lactis*; Mae, *Methanococcus aeolicus*; Mav, *Mycobacterium avium*; Mja, *Methanococcus jannaschii*; Mle, *Mycobacterium leprae*; Mth, *Methanobacterium thermoautotrophicum*; Mtu, *Mycobacterium tuberculosis*; Ncr, *Neurospora crassa*; Ppu, *Porphyra purpurea*; Sav, *Streptomyces avermitilis*; Sce, *Saccharomyces cerevisiae*; Sci, *S. cinnamonensis*; Sma, *Schizosaccharomyces malidevorans*; Spa, *Saccharomyces pastorianus*; Spo, *Schizosaccharomyces pombe*; Ssp, *Synechocystis* sp; Sty3, *Salmonella typhimurium* isozyme III; Tma, *Thermotoga maritima*. The arrows indicate the four positions mutated in the present study and those highlighted in red are residues that are conserved in all sequences except for *E. coli* isozyme II. Those highlighted in yellow are invariably large hydrophobic residues. Panel B shows a molecular model of the *A. thaliana* AHAS regulatory subunit. The structure shown is that of the regulatory domain of *E. coli* threonine deaminase (PDB accession code 1TDJ). This structure was modified by mutation at the eight residues shown using the Swiss-PdbViewer (http://au.expasy.org/spdbv/). The resulting coordinate file was visualised using WebLab Viewer Pro (http://www.accelrys.com/viewer/), which was used to create the final diagram in conjunction with POV-Ray (http://www.povray.org).

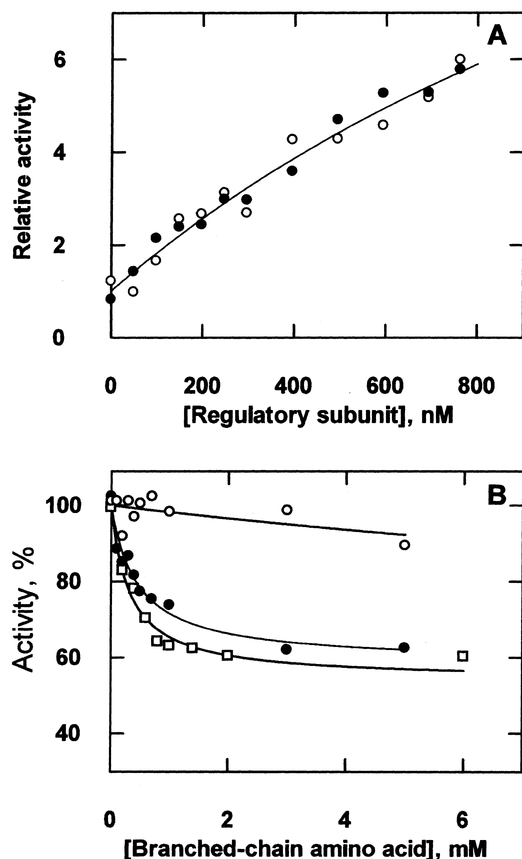


Fig. 2. Kinetic properties of *A. thaliana* AHAS reconstituted using wild-type catalytic subunits and the N113A variant of the regulatory subunit. Panel A shows the effect on activity of reconstitution by varying concentrations of the N113A variant of the regulatory subunit. The different symbols represent two independent experiments and were normalised to a common value of 1.0 for the activity of the catalytic subunit alone. Panel B shows the inhibition of the reconstituted enzyme by leucine alone (●), valine alone (○), or an equimolar mixture of leucine plus valine (□). Data for the effect of isoleucine alone have been omitted for clarity but are similar to those obtained for valine.

tamate (cf. *E. coli* AHAS isoenzyme II) while G331 was mutated to aspartate (cf. *E. coli* AHAS isoenzyme III and *S. cinnamonensis* mutants).

Each mutated regulatory subunit is capable of activating the *A. thaliana* catalytic subunit, as illustrated for the N113A variant in Fig. 2A. Due to the limited solubility of the regulatory subunit it is not possible to achieve 100% reconstitution. However, for all variants, sufficient regulatory subunit was added to stimulate the activity by at least 5-fold. These reconstituted enzymes were then tested for inhibition by each of the branched-chain amino acids, as well as by an equimolar mixture of valine plus leucine (Fig. 2B). The apparent inhibition constants (K_i^{app}) obtained from these experiments are summarised in Table 1. In all cases, saturation with the inhibitor results in some residual activity; the percent inhibition at saturation is also reported in Table 1 and ranges from 15 to 73% of the uninhibited rate. The percent inhibition by valine plus leucine is invariably similar to that obtained by either valine or leucine alone, whichever is greatest.

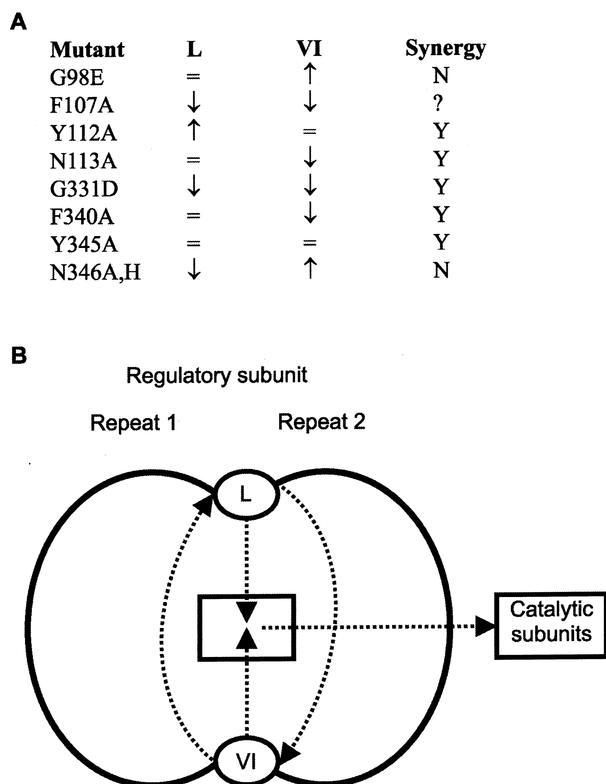
A range of effects was observed varying from complete abolition of inhibition by all branched-chain amino acids (F107A) to an enzyme that is very similar to wild-type (Y345A). In all cases, the effects on valine and isoleucine inhibition are similar, insofar as any changes in the K_i^{app} values are both in the same direction when compared to the wild-type value. However, there is no correlation with the effects on leucine inhibition. For example, the G98E change increases the potency of valine and isoleucine without affecting inhibition by leucine. The Y112A variant exhibits exactly the opposite pattern, increasing the potency of leucine without affecting inhibition by valine and isoleucine. There is a marked asymmetry in the results; for example, the F340A mutation interferes with valine and isoleucine inhibition but has no effect on leucine. If there was symmetry, the F107A mutation would be expected to affect leucine inhibition only whereas it actually abolishes inhibition by all of the branched-chain amino acids.

As we have described previously for AHAS reconstituted with the wild-type regulatory subunit, there is synergy between inhibition by the combination of leucine plus valine. If there was no synergy, an equimolar mixture of these two amino acids would be expected to result in a K_i^{app} value that is the harmonic mean of the values for the two individual amino acids. For wild-type, this harmonic mean is 274 ± 23 μM

Table 1
Inhibition of *A. thaliana* AHAS reconstituted using wild-type catalytic subunits and variants of the regulatory subunit

Regulatory subunit	K_i^{app} (percent inhibition)				
	Leucine (mM)	Isoleucine (mM)	Valine (mM)	Leucine+valine (μM)	Harmonic mean (μM)
Wild-type	0.34 ± 0.03 (63 ± 1)	1.38 ± 0.16 (63 ± 2)	0.23 ± 0.03 (45 ± 3)	12.3 ± 2.3 (59 ± 2)	274 ± 23
Repeat 1					
G98E	0.30 ± 0.03 (19 ± 1)	0.37 ± 0.16 (15 ± 1)	0.033 ± 0.006 (26 ± 1)	139 ± 44 (28 ± 2)	59 ± 10
F107A	NI	NI	NI	NI	> 2 × 10^4
Y112A	0.08 ± 0.01 (42 ± 1)	1.26 ± 0.40 (35 ± 3)	0.26 ± 0.03 (21 ± 1)	19.9 ± 1.7 (47 ± 1)	122 ± 12
N113A	0.45 ± 0.11 (41 ± 3)	NI	NI	323 ± 64 (46 ± 2)	≈ 900
Repeat 2					
G331D	NI	NI	NI	557 ± 132 (50 ± 2)	> 2 × 10^4
F340A	0.39 ± 0.11 (57 ± 2)	3.72 ± 1.76 (73 ± 17)	1.28 ± 0.30 (38 ± 3)	14.7 ± 1.2 (57 ± 4)	598 ± 133
Y345A	0.44 ± 0.08 (72 ± 3)	1.30 ± 0.18 (60 ± 3)	0.12 ± 0.02 (31 ± 1)	10.8 ± 1.9 (69 ± 2)	189 ± 26
N346A	1.11 ± 0.23 (61 ± 7)	0.42 ± 0.08 (40 ± 4)	0.009 ± 0.002 (29 ± 2)	32.9 ± 8.6 (55 ± 4)	17.9 ± 4.0
N346H	1.03 ± 0.16 (51 ± 2)	0.46 ± 0.22 (35 ± 3)	0.020 ± 0.004 (36 ± 1)	45.1 ± 5.1 (54 ± 1)	39.2 ± 7.7

NI, less than 10% inhibition at the highest concentration tested; 5 mM for individual amino acids and 10 mM total for the leucine plus valine combination.



A

Mutant	L	VI	Synergy
G98E	=	↑	N
F107A	↓	↓	?
Y112A	↑	=	Y
N113A	=	↓	Y
G331D	↓	↓	Y
F340A	=	↓	Y
Y345A	=	=	Y
N346A,H	↓	↑	N

Fig. 3. Simplified summary of the inhibition results and a schematic model for the regulatory interactions in *A. thaliana* AHAS. Panel A summarises the effects (Table 1) of the inhibitors. The symbol '=' indicates a K_i^{app} value between 0.5 and 1.5 times that of the wild-type, while '↑' and '↓' denote effects outside this range resulting in increased or decreased inhibition, respectively. The presence (Y) or absence (N) of synergy was estimated by comparing the actual K_i^{app} value for the leucine plus valine combination with the harmonic mean of the individual K_i^{app} values for these two compounds. Panel B shows a schematic model for the regulatory interactions. The bilobed regulatory subunit has separate site binding leucine (site L) or valine/isoleucine (site VI) at the interface between the two domains. The dotted arrows show signalling pathways. Occupancy of either site signals to the other site (curved arrows), resulting in increased affinity for its ligand. Site occupancy also generates signals (vertical arrows) that are transmitted to a common centre (boxed) and then (horizontal arrow) to the catalytic subunits.

(Table 1) compared to the observed value of 12.3 ± 2.3 μM. Table 1 lists these calculated harmonic means from which it can be seen that synergy cannot be detected simply by comparing the K_i^{app} value of a mutant for leucine plus valine with that of the wild-type. For example, N113A has a K_i^{app} value of 323 ± 64 μM that is markedly elevated compared to wild-type. However, the expected value based on no synergy would be approximately 900 μM because valine is not inhibitory. An opposite example is seen for the corresponding mutation in repeat 2 (N346A). In this case, the K_i^{app} value for the combination is not greatly elevated relative to wild-type. However, valine alone is a very strong inhibitor so in fact synergy is abolished in this variant.

A simplified summary of the inhibition results and a schematic model that attempts to explain these data are shown in Fig. 3. The regulatory subunit contains two domains corresponding to the two repeats, with separate binding sites for leucine (site L) and for valine/isoleucine (site VI) at the domain interface. There are separate and reciprocal signalling pathways that promote regulator binding at one site when the other is occupied. In addition, occupancy of either site by its ligand is signalled to a common centre that then triggers inhibition of the catalytic activity. The proposal for this common centre is based on the observation that the extent of inhibition is similar for leucine alone and for the leucine plus valine combination.

The G98E mutation does not affect leucine inhibition, promotes valine/isoleucine binding, and interferes with synergy. These results suggest that this mutation is mimicking site L occupancy and must be switching on the signal from site L to site VI. A similar effect is observed for the two N346 mutants, except that leucine inhibition is somewhat impaired, suggesting that N346 may form part of site L. It is of interest that G98 and N346 fulfil similar roles despite being well separated in the primary sequence and located in different repeats. However, these results are entirely consistent with the molecular model (Fig. 1B), which puts these two residues in close proximity. The complementary pair of mutations, G331D and N113A, also have similar effects to one another except that one (G331D) interferes with leucine inhibition while the other does not; again, this supports the molecular model. However, the effects of these mutations are not complementary to those at the G98/N346 pair. G331D prevents inhibition by any of the amino acids suggesting that it is involved in signal transmission to the catalytic subunits. N113A prevents valine and isoleucine inhibition but does not affect leucine; this would be consistent with either a role in site VI, or in signalling to the common centre. However, there is some evidence of synergy that would favour the explanation that N113 is more involved in signalling to the common centre than having a direct role in site VI.

Changing Y345 to alanine has no detectable effect; however, the corresponding change in repeat 1 (Y112A) promotes leucine binding while retaining some synergy. These results would be consistent with partial activation of the site VI to site L signalling pathway. The final pair of mutations, at F107 and F340, again exhibit a disparate effect. F340A has similar effects to N113A suggesting that it too is involved in signalling to the common centre. In contrast, F107A is completely insensitive to all of the branched-chain amino acids, similar to the results for the G331D variant.

The results presented here are, superficially, somewhat at variance with the model and data for the equivalent asparagine mutation in *E. coli* AHAS isoenzyme III [9]. However, it should be noted that for that enzyme it is a regulatory subunit dimer with two equivalent valine-specific sites that corresponds to the paired repeats of the *A. thaliana* subunit. Therefore, a single site mutation in the bacterial protein should be compared with a double mutant of the plant AHAS. Although we have not constructed this double mutant, it would be predicted that changing both asparagine residues to histidines would affect both sites and the double mutant would be insensitive to all of the branched-chain amino acids. This would agree with the observation that the single asparagine mutation in *E. coli* AHAS isoenzyme III strongly decreases valine inhibition [9].

These mutation studies reveal that the regulation of *A. thaliana* AHAS is far more complex than we originally supposed. A mutation that disrupted site L only would be expected to prevent inhibition by leucine, leave inhibition by valine and isoleucine unaffected, and to abolish synergy.

None of the mutations produces this pattern. Similarly, none of the mutations produces a pattern that is consistent with disruption of site VI only. The mutations mainly affect transmission of site occupancy signals between sites, from one of the sites to the common centre, or to the catalytic subunit.

The model that we have proposed is necessarily speculative in that it is derived from a molecular model based on an entirely different protein. While this model may be broadly correct, it is undoubtedly approximate in detail. Further progress in understanding the precise role of the residues that we have mutated must await experimental determination of the three-dimensional structure of this protein.

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References

[1] Chipman, C., Barak, Z. and Schloss, J.V. (1998) Biochim. Biophys. Acta 1385, 401–419.
[2] Duggleby, R.G. and Pang, S.S. (2000) J. Biochem. Mol. Biol. 33, 1–36.
[3] Duggleby, R.G. (1997) Gene 190, 245–249.
[4] Pang, S.S. and Duggleby, R.G. (1999) Biochemistry 38, 5222–5231.
[5] Hershey, H.P., Schwartz, L.J., Gale, J.P. and Abell, L.M. (1999) Plant Mol. Biol. 40, 795–806.
[6] Lee, Y.-T. and Duggleby, R.G. (2001) Biochemistry 40, 6836–6844.
[7] Durner, J. and Böger, P. (1988) Z. Nat.forsch. 43c, 850–856.
[8] Wessel, P.M., Graciet, E., Douce, R. and Dumas, R. (2000) Biochemistry 39, 15136–15143.
[9] Mendel, S., Elkayam, T., Sella, C., Vinogradov, V., Vyazmensky, M., Chipman, D.M. and Barak, Z. (2001) J. Mol. Biol. 307, 465–477.
[10] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[11] Brøns-Poulsen, J., Petersen, N.E., Hørder, M. and Kristiansen, K. (1998) Mol. Cell. Probes 12, 345–348.
[12] Kopecký, J., Janata, J., Pospíšil, S., Felsberg, J. and Spížek, J. (1999) Biochem. Biophys. Res. Commun. 266, 162–166.