11. R. T. Bakker and P. M. Galton, *Nature* **248**, 168 (1974).
12. D. B. Brinkman and H.-D. Sues, *Palaeontology* **30**, 493 (1987).
13. M. J. Benton, in *Third Symposium on Mesozoic Terrestrial Ecosystems*, W. E. Reif and F. Westphal, Eds. (Attempto Verlag, Tübingen, 1984), pp. 13–18; G. S. Paul, *ibid.*, pp. 175–180; *J. Vertebr. Paleontol.* **4**, 507 (1984); J. Gauthier and K. Padian, in *The Beginnings of Birds*, M. K. Hecht, J. H. Ostrom, G. Viohl, P. Wellnhofer, Eds. (Freunde des Jura-Museums Eichstätt, Eichstätt, 1985), pp. 185–197; F. E. Novas, *J. Paleontol.* **63**, 677 (1989); M. J. Benton, in *The Dinosauria*, D. B. Weishampel, P. Dodson, H. Osmólska, Eds. (Univ. of California Press, Berkeley, 1990), pp. 11–30; F. E. Novas, *Palaeontology* **35**, 51 (1992).
14. J. Gauthier, *Calif. Acad. Sci. Mem.* **8**, 1 (1986).
15. P. C. Sereno, *J. Vertebr. Paleontol.* **11**, 1 (1991); _____ and A. B. Arcucci, *ibid.*, in press.
16. Because the skull and forelimb are not preserved in the dinosaur precursors *Lagosuchus* and *Lagerpeton*, dinosaurian synapomorphies in these skeletal parts may eventually characterize a more inclusive group (pterosaurs and basal crurotarsal archosaurs show the primitive condition). The ungual on the fourth digit of the manus is absent in all dinosaurs except Cretaceous iguanodontian ornithischians such as *Iguanodon* [D. B. Norman, *Inst. R. Sci. Nat. Belgique Mem.* **178**, 1 (1980)], in which it constitutes an evolutionary reversal.
17. Because dinosaur polyphyly and saurischian paraphyly were often assumed at this time, the label "saurischian" did not commit one to a specific phylogenetic arrangement, as is evident in Bakker and Galton's (*11, 18*) more detailed discussion of the phylogenetic position of *Herrerasaurus* and *Staurikosaurus* and the origins of Ornithischia and Sauropodomorpha.
18. H.-D. Sues, in *The Dinosauria*, D. B. Weishampel, P. Dodson, H. Osmólska, Eds. (Univ. of California Press, Berkeley, 1990), pp. 143–147.
19. J. F. Bonaparte, *Opera Lilloana* **22**, 1 (1972). The tetanurian status of these fragmentary theropod remains is suggested by the flange-shaped anterior trochanter, slender fibula, and the apparently thin ascending process of the astragalus. Well-preserved ceratosaurians are also present by the late Carnian or Norian (*6, 25*).
20. The age of divergence between sister groups must be as early as, or earlier than, the first record of either sister group. The presence of Prosauropoda thus indicates that its sister taxon Sauropoda was also present. "Ghost" sister lineages may not yet have acquired all of the synapomorphies that unite known members of the sister taxon [W. Hennig, *Phylogenetic Systematics* (Univ. of Illinois Press, Urbana, 1966), pp. 163–169; M. A. Norell, in *Extinction and Phylogeny*, M. J. Novacek and Q. Wheeler, Eds. (Columbia Univ. Press, New York, 1992)].
21. J. F. Bonaparte, *Ameghiniana* **16**, 217 (1979); P. Stipanicíc and J. F. Bonaparte, *II Simp. Geol. Argent.* **1**, 523 (1979).
22. R. R. Rogers *et al.*, paper presented at the Fifth North American Paleontological Convention, Chicago, June 1992.
23. D. H. Erwin, *Hist. Biol.* **6**, 133 (1992).
24. Seasonality is suggested by marked growth variation in fossil conifer wood.
25. Modified from E. H. Colbert, *Mus. N. Ariz. Bull.* **57**, 1 (1989).
26. Based on F. von Huene, *Geol. Palaeontolog. Abh. N. F.* **15**, 129 (1926).
27. Based on R. A. Thulborn, [*Palaeontology* **15**, 29 (1972)] and P. C. Sereno, [*J. Vertebr. Paleontol.* **11**, 168 (1991)] with portions of the sacrum reconstructed as seen in *Hypsilophodon* and *Psittacosaurus*.
28. Supported by NSF research grant BSR 8722586, The David and Lucile Packard Foundation, National Geographic Society, and the Donors of The Petroleum Research Fund administered by the American Chemical Society (grant ACS-PRF 22637-G8) (P.C.S.). We thank W. Amaral, W. Simpson, and C. Vanderslice for fossil preparation and C. Abraczinskas for execution of the finished illustrations. We also thank A. Arcucci, R. Blob, M. Carrano, C. Forster, R. Rogers and H.-D. Sues for comments on the manuscript and A. Monetta and C. Treo for loan of fossil materials.

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Cooperativity Induced by a Single Mutation at the Subunit Interface of a Dimeric Enzyme: Glutathione Reductase

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When glycine^{418} of *Escherichia coli* glutathione reductase, which is in a closely packed region of the dimer interface, is replaced with a bulky tryptophan residue, the enzyme becomes highly cooperative (Hill coefficient 1.76) for glutathione binding. The cooperativity is lost when the mutant subunit is hybridized with a wild-type subunit to create a heterodimer. The mutation appears to disrupt atomic packing at the dimer interface, which induces a change of kinetic mechanism. A single mutation in a region of the protein remote from the active site can thus act as a molecular switch to confer cooperativity on an enzyme.

Many enzymes are oligomeric; of these, some exhibit standard Michaelis-Menten kinetics and others display homotropic or heterotropic cooperativity in ligand binding and a characteristically sigmoidal dependence of reaction velocity on substrate concentration. The two principal models advanced to explain cooperative behavior (*1, 2*) rely on ligand-induced conformational changes in the protein, the effects of which are transmitted across a domain or subunit interface. Some of these ideas have recently been tested by directed mutagenesis (*3, 4*). Cooperativity can be eliminated: For example, replacement of key residues involved in the binding of fructose 6-phosphate in the active site of *Escherichia coli* phosphofructokinase leads to loss in cooperative behavior in the enzyme tetramer (*5*). Similarly, the elimination of certain interactions between the aspartate and carbamoyl phosphate domains of the catalytic subunit of *E. coli* aspartate transcarbamoylase causes it to lose both catalytic activity and cooperativity in binding aspartate (*6*).

Conferral of cooperative behavior on an enzyme has also been reported. Replacement of an active-site Arg residue with Gly in *E. coli* ornithine transcarbamoylase creates an enzyme of reduced catalytic efficiency but one that exhibits cooperative responses to both substrates (*7*). Likewise, replacement of an active-site Arg residue with Gly in the noncooperative aspartate transcarbamoylase of *Bacillus subtilis* converts it into a cooperative enzyme (*8*).

We have been able to confer cooperativity on an enzyme, *E. coli* glutathione reductase (E.C. 1.6.4.2), by manipulation of the subunit interface away from the active site. This dimeric enzyme is a member of the important family of flavoprotein disulfide oxidoreductases (*9, 10*). Glutathione reductase catalyzes the following reaction:

$$H^+ + NADPH + GSSG = NADP^+ + 2GSH \quad (1)$$

where NADPH is the reduced form of nicotinamide adenosine dinucleotide phosphate ($NADP^+$) and GSH and GSSG are the reduced and oxidized forms of glutathione, respectively. The crystal structure of the human enzyme at 1.5 Å resolution (*11*) serves as the reference point for the rest of the family (*12–15*). The primary (*16*) and three-dimensional (*17*) structures of *E. coli* glutathione reductase are similar to those of the human enzyme, enabling the cloned gene (*gor*) for *E. coli* glutathione reductase to be used for protein engineering experiments, notable among them the insertion of an intersubunit disulfide bridge (*18*), investigation of residues critical to the catalytic mechanism (*19–21*), and switches of the coenzyme specificity from NADPH to NADH (*22*) and the substrate specificity from glutathione toward trypanothione (*23*).

The enzyme normally exhibits a ping-pong kinetic mechanism (*9, 24, 25*), but replacement of a single residue (Tyr^{177}) in the NADPH binding site changes it to ordered sequential (*19*). No homotropic or heterotropic cooperativity has been observed in the binding of NADPH or glutathione. The binding sites for NADPH and glutathione are physically distinct and are separated by the isoalloxazine ring of the enzyme-bound flavin-adenine dinucle-

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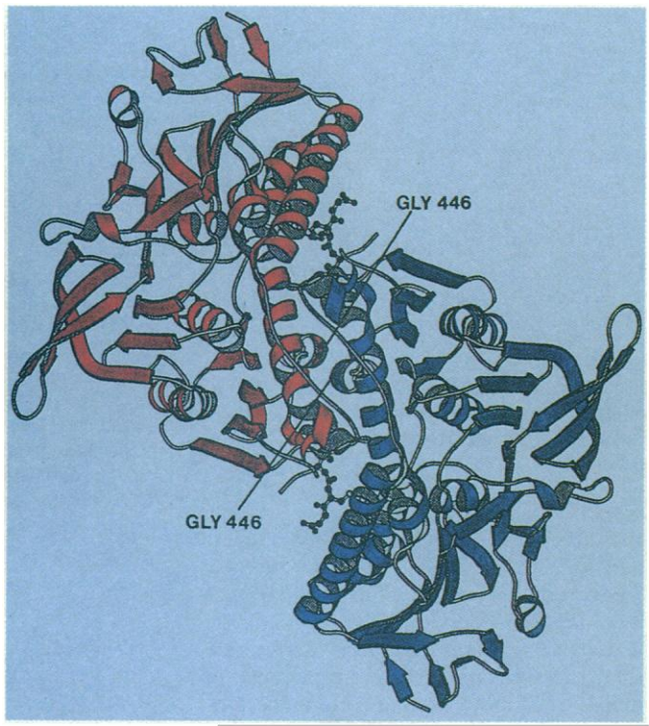

Fig. 1. Three-dimensional structure of human glutathione reductase. The coordinates were provided by G. E. Schulz, and the representation of the structure was generated with the program MOLSCRIPT (*29*). Two glutathione molecules are shown bound across the active site clefts between the two subunits. The positions of the Gly^{446} (Gly^{418} in *E. coli* glutathione reductase) residues on two parallel α helices contributed by opposing subunits are indicated. They are located on the dyad axis of symmetry.

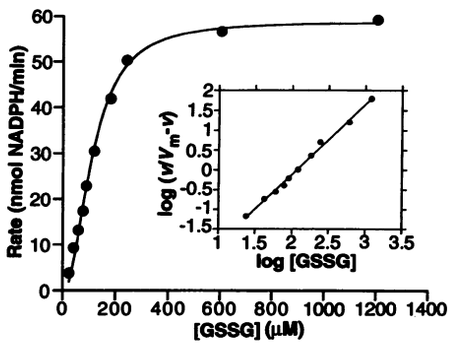

Fig. 2. Reaction rate as a function of glutathione concentration for the reaction catalyzed by G418W (homodimer) *E. coli* glutathione reductase. The enzyme was assayed in the direction of GSSG reduction at a fixed concentration (100 μM) of NADPH (*19, 22*). Inset: Hill plot of the data, interaction coefficient 1.76. The data were fitted by nonlinear least-squares regression analysis, and the lines of best fit are shown.

Table 1. Specific catalytic activities and kinetic parameters of wild-type and mutant forms of *E. coli* glutathione reductase. The enzymes were assayed, and kinetic parameters were measured as described previously (*19, 22*). All of the catalytic rate constant k_{cat} and K_m values given are apparent values (*22*). The true values of K_m[NADPH] and k_{cat} for the wt enzyme are 38 ± 4 μM and 36,000 ± 2,600 min^{-1}, respectively (*19*). The apparent values were measured by varying the NADPH concentration at a fixed concentration (1.2 mM) of GSSG. Glutathione reductase is subject to substrate inhibition; hence the true k_{cat} is higher (twofold) than the apparent k_{cat}.

Enzyme	K_m[GSSG] (μM)	K_m[NADPH] (μM)	k_{cat} (min^{-1})	Hill coefficient
Wild-type	97 ± 12	22 ± 2	16,000 ± 350	1.00
G418W homodimer	*	41 ± 5	8,600 ± 160	1.76
G418W/WT-Arm heterodimer	43 ± 6†	44 ± 4	23,000 ± 810	1.06

*The values of K_m[GSSG] and k_{cat} at 400 μM NADPH were 311 ± 33 μM and 10,200 ± 900 min^{-1}. †The value of K_m[GSSG] for the heterodimer is an apparent one, measured at 100 μM NADPH.

otide (FAD) and a redox-active disulfide bridge of the protein (*11, 24*). The binding sites for glutathione reside in clefts formed by the dimer interface (related by the twofold axis) and are composed of amino acid side chains contributed by both subunits, making it inconceivable that monomers of the enzyme could be active in glutathione reduction.

The dimer interface has two structurally well-defined regions (*11*): The upper region, formed by part of a distinct interface domain, is one of the least flexible sections of the protein whereas the lower region, formed by helical extensions from the FAD binding domains, is one of the most flexible. In the upper region, Gly^{418} (Gly^{446} in the human enzyme) is in a densely packed part of the enzyme (Fig. 1). Replacement of Gly^{418} with a Trp residue (G418W) should substantially perturb the close interactions with $Phe^{419'}$ (contributed by the opposing subunit at the dimer interface) [Gly^{446} and $Phe^{447'}$ in the human enzyme (*11*)].

Site-directed mutagenesis of the Gly^{418} site in the *E. coli gor* gene in the vectors K19gor3′δEco RI or K19gorEco RI and verification of the products were carried out as described elsewhere (*20*). Wild-type (wt) and mutant glutathione reductases were purified from the *gor*-deletion strain (NS3) of *E. coli* (*20*) or the proteinase-deficient strain (NA33) of *E. coli* (*26*) transformed with the appropriate expression plasmid. Steady-state kinetic assays and the measurement of kinetic parameters were carried out as described previously (*19, 22*).

The G418W mutant glutathione reductase was purified from *E. coli* strain NS3 under the conditions described for the wt enzyme (*20*). During gel filtration through Superose-12 (at protein concentrations as low as 50 μg/ml) it was eluted at the same position as the wt protein, consistent with a molecular weight of ~100 kD and a normal dimeric structure. The mutant enzyme was also catalytically active: at a fixed concentration (1.2 mM) of glutathione, the kinetics obeyed the usual hyperbolic rate equation, with an apparent Michaelis constant (K_m) for NADPH similar to that of wt glutathione reductase (Table 1). The apparent catalytic rate constant (k_{cat}) (8,600 min^{-1}) is approximately one-half the apparent k_{cat} (16,000 min^{-1}) measured for the wt enzyme under the same conditions (*22*).

However, when the concentration of glutathione was varied at a fixed nonsaturating concentration (100 μM) of NADPH, a hyperbolic rate equation was not followed (Fig. 2). A good fit was obtained to a rate equation expressing positive cooperativity between the two glutathione binding sites (*27*):

$$v = V_{max}\left(\frac{\frac{S}{K_m} + \frac{S^2}{aK_m^2}}{1 + \frac{2S}{K_m} + \frac{S^2}{aK_m^2}}\right) \quad (2)$$

S is the substrate concentration. The cooperativity was strong, as evidenced by a Hill coefficient of 1.76 (Fig. 2, inset). A reliable value of K_m cannot be estimated because K_m and the interaction factor *a* are not independent variables. Cooperativity with glutathione was lost when the fixed concentration of NADPH was raised to a saturating concentration (400 μM) and a normal hyperbolic rate equation was then followed. It is clear that the kinetic mechanism has changed, perhaps to one involving ternary

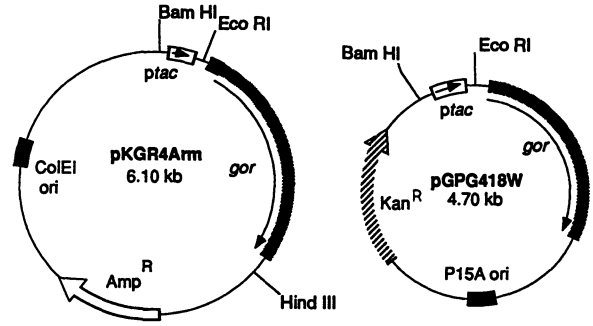

Fig. 3. Expression plasmids for expressing the G418W and WT-Arm forms of *E. coli* glutathione reductase. The construction of plasmid pKGR4Arm has been described (*26*). It encodes wt glutathione reductase carrying the additional sequence RRRRR-IEGR- (a penta-arginine segment plus factor Xa cleavage site R, Arg; I, Ile; E, Glu; and G, Gly) at the NH_2-terminus of the protein. Plasmid pGPG418W was constructed as follows: The G418W mutation was created by mutagenesis with the bacteriophage K19gorEco RI DNA (*20*). The mutant gene was subcloned as an Eco RI–Hind III fragment into the expression vector pKK223-3 (*20*) to yield plasmid pKGR3G418W. The transcriptional unit from this plasmid was excised by digestion with Bam HI and Nsi I and was subcloned into plasmid pGP1-2 cut with Bam HI and Pst I (*28*). The resultant construct is designated plasmid pGPG418W.

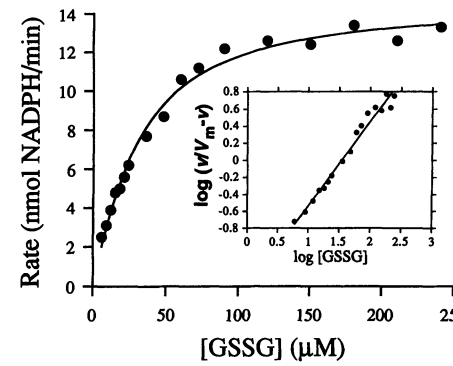

Fig. 4. Reaction rate as a function of glutathione concentration for the reaction catalyzed by G418W/WT-Arm (heterodimer) *E. coli* glutathione reductase. The enzyme was assayed, and the data were processed as in Fig. 2. Inset: Hill plot of the data, interaction coefficient 1.06.

enzyme complex (E.NADPH.GSSG) formation through two different routes (*27*), but the precise mechanism remains to be established.

We tested the effect of replacing only one of the Gly^{418} residues in the dimer by creating a heterodimer, in which only one subunit carried the mutation. Two *gor* genes from different plasmids (Fig. 3) were expressed in the same *E. coli* NA33 cell: one (pGPG418W) encoded a glutathione reductase chain carrying the G418W mutation; the other (pKGR4Arm) encoded a wt subunit that contained a penta-arginine extension at its NH_2-terminus (wt-arm). We have shown (*26*, *28*) that this leads to the formation of hybrid dimers in vivo, that the positively charged NH_2-terminal extension makes possible the subsequent separation of the hybrid enzyme from the two parental forms in vitro, and that the NH_2-terminal extension is otherwise benign and without effect on the kinetic properties of glutathione reductase.

At a fixed concentration (1.2 mM) of glutathione, the kinetics of the purified G418W/WT-Arm hybrid enzyme were normal hyperbolic, with an apparent K_m for NADPH similar to that found for the wt enzyme and the G418W homodimer (Table 1). When the NADPH concentration was held fixed at 100 μM and the glutathione concentration was varied, the kinetic data could be fitted equally well to a rectangular hyperbola or to a sigmoid rate equation with a Hill coefficient of only 1.06 (Fig. 4). Thus, if there is any residual cooperativity between the glutathione binding sites, it is weak. The apparent K_m for glutathione (43 μM) is similar to the true K_m (97 μM) measured for the wt enzyme (Table 1).

Our choice of the G418W mutation was predicated on the assumption that it would lead to significant disruption of the glutathione reductase dimer interface. As a simple test of this, the thermal stabilities of the wt enzyme, the G418W homodimer, and the G418W/WT-Arm heterodimer, were studied (Fig. 5). The G418W homodimer was found to lose its catalytic activity at a substantially lower temperature than the wt enzyme, and the presence of GSSG (120 mM) exerted little or no protective effect. The G418W/WT-Arm heterodimer was intermediate in its response to thermal denaturation. This result strongly suggests that the structure of the G418W mutant enzyme has been disrupted and that the disruption is relieved, at least in part, when the G418W mutant subunit is allowed to hybridize with a wt subunit.

Disruption of the closely packed interior of *E. coli* glutathione reductase by the substitution of bulky Trp residues for the two Gly^{418} residues on opposite sides of the subunit interface (Fig. 1) is not enough to prevent dimer formation, as judged by gel filtration, although we cannot wholly exclude a monomer-dimer equilibrium at low protein concentrations, with glutathione favoring the formation of dimer. It is enough, however, to cause a change of kinetic mechanism, with concomitant acquisition of cooperativity with respect to glutathione. Given the separation of the NADPH and glutathione binding sites, this occurs without significant apparent effect on the K_m for NADPH or substantial loss of catalytic activity. The results with the G418W/WT-Arm heterodimer, in which the structural disruption is substantially relieved, as expected, are fully in accord with this explanation.

In these experiments we have demonstrated that cooperativity can be conferred on an enzyme by a single mutation in what is at first sight a mechanistically dull part of the structure, namely, the subunit interface. With *E. coli* glutathione reductase, conferral of cooperativity has been purchased at the expense of some loss of thermal stability of the protein. Nonetheless, it is a clear indication that the molecular switch need be only small, something that is easily acquired during evolution of a metabolic pathway.

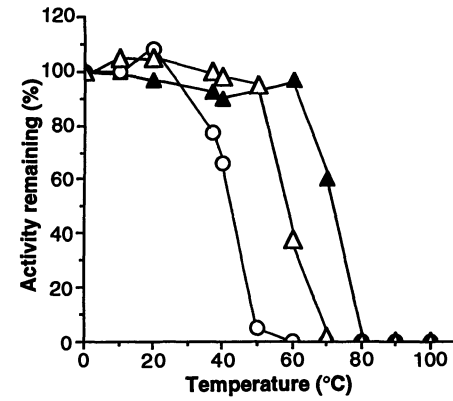

Fig. 5. Thermal stability of wt and mutant *E. coli* glutathione reductases. Enzyme activities were determined in the presence of 100 μM NADPH and 1.2 mM GSSG after the enzymes were incubated in the assay buffer at the desired temperature for 10 min as previously described (*19*); (▲), wt glutathione reductase; (△), G418W/WT-Arm heterodimer glutathione reductase; and (○), G418W homodimer glutathione reductase.

REFERENCES AND NOTES

1. J. Monod, J. Wyman, J. P. Changeux, *J. Mol. Biol.* **12**, 88 (1965).
2. D. E. Koshland, Jr., G. Nemethy, D. Filmer, *Biochemistry* **5**, 365 (1966).
3. F. Van Vliet *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9180 (1991).
4. S. R. Wente and H. K. Schachman, *J. Biol. Chem.* **266**, 20833 (1991).
5. S. A. Berger and P. R. Evans, *Nature* **343**, 575 (1990).
6. N. J. Dembowski, C. J. Newton, E. R. Kantrowitz, *Biochemistry* **29**, 3716 (1990).
7. L. C. Kuo, I. Zambidis, C. Caron, *Science* **245**, 522 (1989).
8. J. W. Stebbins and E. R. Kantrowitz, *Biochemistry* **31**, 2328 (1992).
9. C. H. Williams, Jr., in *Chemistry and Biochemistry of Flavoproteins*, F. Müller, Ed. (CRC Press, Boca

Raton, FL, 1992), vol. III, pp. 121–211.
10. R. N. Perham, N. S. Scrutton, A. Berry, *BioEssays* **13**, 515 (1991).
11. P. A. Karplus and G. E. Schulz, *J. Mol. Biol.* **195**, 701 (1987).
12. A. Mattevi, A. J. Schierbeek, W. G. J. Hol, *ibid.* **220**, 975 (1991).
13. N. Schiering *et al.*, *Nature* **352**, 168 (1991).
14. J. Kuriyan *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8764 (1991).
15. J. Kuriyan *et al.*, *Nature* **352**, 172 (1991).
16. S. Greer and R. N. Perham, *Biochemistry* **25**, 2736 (1986).
17. U. Ermler and G. E. Schulz, *Proteins* **9**, 174 (1991).
18. N. S. Scrutton, A. Berry, R. N. Perham, *FEBS Lett.* **241**, 46 (1988).
19. A. Berry, N. S. Scrutton, R. N. Perham, *Biochemistry* **28**, 1264 (1989).
20. M. P. Deonarain, A. Berry, N. S. Scrutton, R. N. Perham, *ibid.*, p. 9601.
21. M. P. Deonarain, N. S. Scrutton, A. Berry, R. N. Perham, *Proc. R. Soc. London Ser. B* **241**, 179 (1990).
22. N. S. Scrutton, A. Berry, R. N. Perham, *Nature* **343**, 38 (1990).
23. G. B. Henderson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8769 (1991).
24. E. F. Pai and G. E. Schulz, *J. Mol. Biol.* **258**, 1751 (1983).
25. K. K. Wong, M. A. Vanoni, J. S. Blanchard, *Biochemistry* **27**, 7091 (1988).
26. M. P. Deonarain, N. S. Scrutton, R. N. Perham, *ibid.* **31**, 1491 (1992).
27. I. H. Segel, *Enzyme Kinetics* (Wiley-Interscience, New York, 1975), pp. 460–461.
28. M. P. Deonarain, N. S. Scrutton, R. N. Perham, *Biochemistry* **31**, 1498 (1992).
29. P. J. Kraulis, *J. Appl. Crystallogr.* **24**, 946 (1991).
30. Supported by the Science and Engineering Research Council (SERC) and the Cambridge Centre for Molecular Recognition, the Royal Society and St. John's College, Cambridge. M.P.D. was supported by a research studentship from the SERC and a benefactors' research scholarship from St. John's College. N.S.S. was a research fellow of the Royal Commission for the Exhibition of 1851 and a research fellow of St. John's College, Cambridge. A.B. is a Royal Society 1983 university research fellow. We thank P. J. F. Henderson for discussion. The mutagenic oligonucleotide 5′-ACGAAATGTTGCAGTGGTTCGCGG-3′ was synthesized (Biosearch Cyclone) by M. Weldon (Department of Biochemistry).

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In Vitro Transcriptional Activation by a Metabolic Intermediate: Activation by Leu3 Depends on α-Isopropylmalate

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In the absence of the leucine biosynthetic precursor α-isopropylmalate (α-IPM), the yeast LEU3 protein (Leu3p) binds DNA and acts as a transcriptional repressor in an in vitro extract. Addition of α-IPM resulted in a dramatic increase in Leu3p-dependent transcription. The presence of α-IPM was also required for Leu3p to compete effectively with another transcriptional activator, GAL4/VP16, for limiting transcription factors. Therefore, the addition of α-IPM appears to convert a transcriptional repressor into an activator. This represents an example in eukaryotes of direct transcriptional regulation by a small effector molecule.

An intricate feedback loop regulates leucine biosynthesis in fungi (*1*). Multiple controls of the first step, the synthesis of α-isopropylmalate (α-IPM), and the participation of a transcriptional activator are central features of the pathway. In yeast, the LEU4-encoded α-IPM synthase is subject to feedback inhibition by leucine, and the LEU4 gene is transcriptionally regulated by both GCN4 and LEU3 (*2*). The LEU3 gene product (Leu3p) is a 100-kD DNA binding protein that belongs to the $Zn(II)_2Cys_6$ binuclear cluster family of eukaryotic transcriptional activators (*3*). Leu3p activates transcription of the LEU1, LEU2, and LEU4 genes (*4*), and its binding site (UAS_{LEU}) is also found upstream of the ILV2, ILV5, and GDH1 genes of yeast (*5*). Genetic evidence suggests that activation by Leu3p requires the presence of α-IPM to complete the regulatory loop (*4*).

To analyze the components required for regulation, we have reconstituted Leu3p-dependent transcriptional activation in vitro. We prepared active whole cell transcription extracts (*6*) from LEU3 null, wild-type, and overexpressing yeast strains (*7*) and measured the amount of Leu3p by means of an electrophoretic mobility shift assay (*3*, *4*). The extracts from wild-type cells contained relatively little Leu3p, but high amounts of UAS_{LEU} binding activity were detected in extracts from the Leu3p-overproducing cells (*8*). The mobility shift obtained with the transcription extracts was indistinguishable from that of highly purified Leu3p, indicating that no auxiliary factors had been lost during the extensive purification of the protein (*8*, *9*). Complex formation by the extracts and by purified Leu3p was unaffected by α-IPM (*4*, *8*).

In vitro transcription reactions were performed as described (*6*). Templates contained various binding sites upstream of the CYC1 TATA box linked to a "G-less cassette," allowing the simple analysis of specific ribonuclease T1–resistant transcripts (*6*). Two templates, distinguishable by the lengths of their G-less cassettes, were used simultaneously to judge relative transcription efficiency. Transcriptional activation was attained with templates that contained either one or two UAS_{LEU} elements (Fig. 1). Activation was clearly dependent on the presence and concentration of Leu3p and absolutely required the addition of α-IPM. Tenfold to 20-fold activation was obtained with the extract from Leu3p-overproducing cells. Neither Leu3p nor α-IPM had any effect on transcription from the control template lacking the UAS_{LEU} sequence (Fig. 1).

We determined transcriptional activation as a function of α-IPM concentration (Fig. 2) (*7*). Saturation curves were identical over a twofold range of extract con-

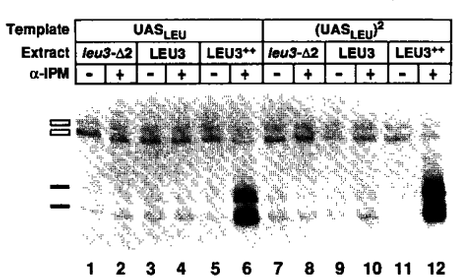

Fig. 1. Transcriptional activation from the UAS_{LEU} requires both Leu3p and α-IPM. In vitro transcription was carried out as described (*6*) with 1.2 μg of pUC18 and 0.3 ng of each template per 30-μl reaction. The longer pair of transcripts (open bars) are derived from a UAS−, basal template bearing the CYC1 TATA box linked to a G-less cassette (*6*). The shorter pair of transcripts (closed bars) is derived from plasmids with a 3′ truncation of the G-less cassette and with one (lanes 1 to 6) or two (lanes 7 to 12) UAS_{LEU} elements upstream of the TATA box (*14*). The α-IPM (natural isomer) (*15*) was added to a final concentration of 0.5 mM to reactions whose products were visualized in even-numbered lanes. Lanes 1, 2, 7, and 8, extract from the *leu3-Δ2* strain at a final concentration of 2 mg of protein per milliliter. Lanes 3, 4, 9, and 10, extract from the wild-type strain at a final concentration of 3 mg of protein per milliliter. Lanes 5, 6, 11, and 12, extract from the Leu3p-overproducing strain at a final concentration of 3 mg of protein per milliliter.

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