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Chemistry

In vivo catalysis of a metabolically essential reaction by an antibody

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ABSTRACT **We have established a growth selection requirement for a catalytic antibody with modest chorismate mutase activity. Conversion of (−)-chorismate into prephenate is the key step in the biosynthesis of the aromatic amino acids tyrosine and phenylalanine. Strains of the yeast *Saccharomyces cerevisiae* containing an insertion mutation in the structural gene for the enzyme chorismate mutase (EC 5.4.99.5) require exogenous supplements of these two amino acids for efficient growth. Intracellular expression of the heterologous antibody catalyst in one such strain, identified by random mutagenesis and genetic selection, provides a substantial growth advantage under auxotrophic conditions; complementation was not observed with an unrelated esterolytic antibody. In addition to demonstrating that tailored immunoglobulin catalysts can carry out vital biochemical reactions *in vivo*, these experiments provide a powerful selection assay for identifying genetic changes within the antibody molecule itself that augment chemical efficiency.**

Antibodies generated against appropriately designed transition state analogs catalyze a wide variety of chemical transformations (1, 2). Like naturally occurring enzymes, catalytic antibodies achieve substantial rate accelerations with high regio- and stereoselectivity under mild aqueous conditions. As both the mechanism and recognition properties of immunoglobulin catalysts are defined by the structure of the immunizing antigen, this technology provides a potentially general method for preparing tailored enzyme-like molecules on demand for practical applications in chemistry, biology, and medicine. For example, the specificity and biocompatibility of catalytic antibodies suggest that they might be useful for effecting important chemical transformations *in vivo*. Here we demonstrate the feasibility of this proposition by showing that an antibody with modest chorismate mutase (EC 5.4.99.5) activity (3) can function inside a yeast cell lacking the natural enzyme and confer a growth advantage to its host by virtue of its catalytic activity.

MATERIALS AND METHODS

Antibody Expression. The genes encoding the catalytic antibody 1F7 were expressed as described (4) using plasmid pABZ260 and the chorismate mutase-deficient *Saccharomyces cerevisiae* strain YT-4Ca (*MATa*, *aro7*::*HIS3*, *leu2*, *ura3*). Plasmid pABZ265, carrying the genes encoding the esterolytic catalytic antibody 6D4 (5, 6), served as a control (see Fig. 2).

***In Vitro* Mutagenesis and Selection.** Cells transformed with pABZ260 were subjected to random mutagenesis with ethyl methanesulfonate according to standard procedures (7). Survival after mutagenesis was ≈20%. Approximately 5×10^9 mutagenized cells were spread on solid yeast extract/peptone/dextrose medium, allowed to grow at 30°C for 2 days, and replicated onto tyrosine dropout plates containing 2% galactose. Colonies that appeared under selective conditions were picked and plated on phenylalanine dropout plates containing 2% galactose in order to isolate single colonies. Purified mutants were tested for the presence of plasmid by colony hybridization (8) and for expression of functional antibody by ELISA (9).

Analysis of Mutants. Individual mutants were tested for segregation of the Phe^+ growth phenotype with the antibody-bearing plasmid (7). Segregants were identified by patching cells on 1% yeast extract/2% peptone/2% galactose plates to allow plasmid loss, then streaking for single colonies, and printing on synthetic minimal plates with 2% galactose but lacking either leucine or phenylalanine. Auxotrophy for leucine, phenylalanine, tyrosine, and uracil was verified on the corresponding selection plates. Plasmid DNA was recovered from individual mutants by transforming *Escherichia coli* strain DH5α with total yeast DNA and selecting for ampicillin-resistant (Amp^r) transformants. DNA samples were then characterized by sizing gel electrophoresis and restriction mapping. Mutagenized and unmutagenized plasmids were reintroduced into the original and mutagenized mutase-negative yeast strains, and the resulting transformants were assayed under selective conditions in liquid culture and on agar plates.

RESULTS AND DISCUSSION

Carlson (10) demonstrated a number of years ago that functional antibody molecules can be expressed cytoplasmically in yeast to alter phenotype. An engineered strain of *S. cerevisiae* containing an antibody directed against yeast alcohol dehydrogenase was shown to exhibit increased growth on allyl alcohol due to limited neutralization of the target enzyme by the immunoglobulin. We wondered whether it would be possible to extend this work and exploit antibodies as tailored intracellular catalysts rather than simply as receptors.

Our efforts to date have focused on the shikimate pathway for the biosynthesis of aromatic amino acids in yeast (11). The conversion of (−)-chorismate into prephenate, a formal aliphatic Claisen rearrangement, is the committed step toward production of phenylalanine and tyrosine in this scheme (Fig. 1). The enzyme chorismate mutase enhances the rate of this reaction by $>10^6$-fold over the uncatalyzed thermal process (12). In *S. cerevisiae*, chorismate mutase is a monofunctional, cytoplasmic protein consisting of two identical subunits (M_r, 30,000). Its structural gene, *ARO7*, has been cloned, and mutations in it confer auxotrophy for tyrosine and phenylalanine (13, 14).

Antibodies with chorismate mutase activity have been elicited with a compound that mimics the putative high-energy transition state for the rearrangement of chorismate into prephenate (3, 15, 16). We recently expressed the genes encoding the Fab protein fragment of one of these antibodies (1F7) in the engineered yeast strain YT-4Ca, which lacks natural chorismate mutase activity (4). The antibody gene

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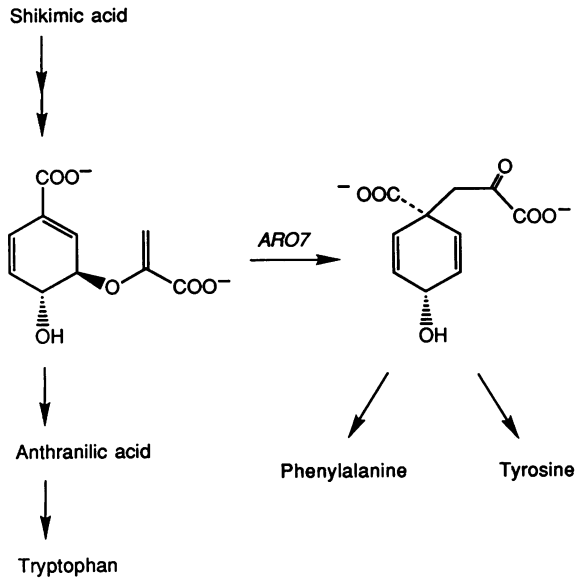

FIG. 1. Shikimate pathway for biosynthesis of aromatic amino acids in yeast. *ARO7* encodes the enzyme chorismate mutase, which promotes the rearrangement of chorismate into prephenate.

segments (minus the signal sequences) were inserted into an inducible expression vector under the control of the *GAL1–GAL10* promoters (pABZ260; Fig. 2). Galactose induction of cells transformed with pABZ260 produced high levels of correctly assembled Fab in the host's cytoplasm. We estimated that the intracellular concentration of 1F7 corresponded to 0.1% of the total cellular protein in these transformants, ≈10 times higher than the concentration of chorismate mutase in wild-type cells (12). However, the Fab isolated from yeast exhibited the same specific activity *in vitro* as the hybridoma-derived immunoglobulin—i.e., a 200-fold rate acceleration over background (3)—and rescue of the host's chorismate mutase deficiency was not observed (4).

Successful complementation of a genetic defect with a heterologous antibody catalyst is likely to depend on many

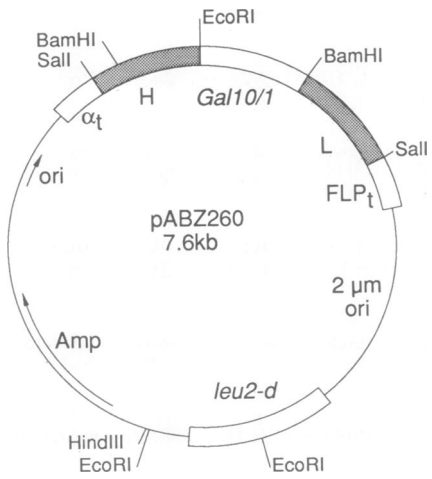

FIG. 2. High copy number yeast plasmid for expression of the Fab protein fragment of the chorismate mutase antibody 1F7 (pABZ260). The plasmid was constructed as described (4); H and L represent truncated heavy- and light-chain antibody coding genes of 1F7, respectively. The *leu2-d* gene serves as a selectable marker, and α_t and FLP_t correspond to the 3′-transcriptional termination signals of the MFα1 and FLP genes. Plasmid pABZ265 is an identical construct except that H and L are replaced by the respective truncated heavy- and light-chain coding genes for the esterolytic antibody 6D4 (5, 6).

interrelated factors, including catalytic turnover, level of expression, protein assembly and half-life, and cellular localization. To isolate a chorismate mutase-deficient yeast strain capable of being rescued by 1F7 we have used mutagenesis with ethyl methanesulfonate and genetic selection. A screen of 10^9 mutants generated by treating antibody-harboring YT-4Ca cells with ethyl methanesulfonate (7) yielded 298 colonies that grew under auxotrophic conditions. One of these mutants, designated 351m::1F7*, was characterized in some detail and shown to require the activity of 1F7 for growth in the absence of exogenous tyrosine and phenylalanine.

Several lines of evidence demonstrate that the chorismate mutase antibody plays an essential metabolic role in 351m::1F7*. First, the Phe^+, Tyr^+ phenotype segregates with the antibody-bearing plasmid. Strain 351m, obtained from 351m::1F7* after segregation and lacking the antibody-encoding plasmid, grows poorly under auxotrophic conditions. In liquid medium without phenylalanine, for example, the difference in growth rate between 351m::1F7* and 351m is striking (Fig. 3). The antibody-containing strain grows into stationary phase at 65 hr, achieving a final cell density of 8.8 × 10^7 cells per ml. Its doubling time in logarithmic phase is ≈6 hr. In contrast, strain 351m only reached a cell density of 2.5 × 10^7 cells per ml after 72 hr and did not exhibit logarithmic-phase growth. Qualitatively similar results were obtained on agar selection plates (Fig. 4*A*).

The second observation supporting the functional significance of intracellularly expressed 1F7 is that 351m::1F7* requires galactose as a carbon source for growth under selective conditions (Fig. 4*A*). When glycerol is used instead, no selective growth advantage over strain 351m is observed (Fig. 4*B*). Glycerol itself is not harmful to the cells, as both strains grow normally on glycerol when phenylalanine and tyrosine are supplied (Fig. 4*C*). These results indicate that the *GAL1–GAL10* promoters controlling transcription of the antibody-encoding genes must be activated in order to achieve complementation. We confirmed by an independent ELISA that antibody protein is produced in cells containing the (1F7)Fab expression plasmid only upon galactose induction.

Finally, and most importantly, we were able to show that serial transformation of the mutant yeast strain allowed reconstitution of its defective shikimate pathway. Yeast cells, transformed with either the plasmid recovered from the 351m::1F7* cells (351m::1F7*^{re}) or the original 1F7 expres-

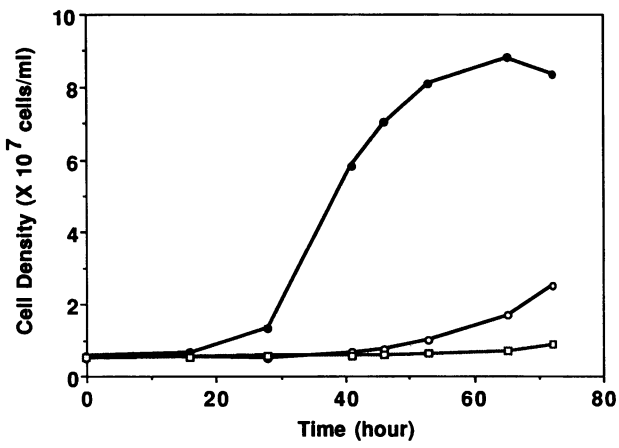

FIG. 3. Growth curves for yeast strains 351m::1F7* (●), 351m (○), and YT-4Ca (□) under selective conditions. Each strain was grown at 30°C in 5 ml of liquid medium containing 2% galactose but lacking phenylalanine from a starting concentration of 5 × 10^6 cells per ml. Cell density was determined by counting cells with a hemacytometer.

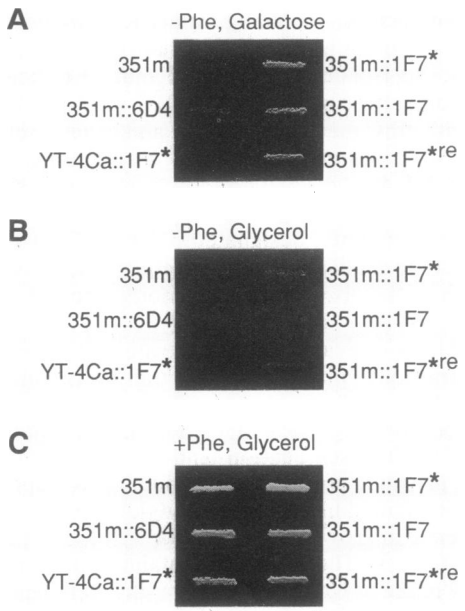

FIG. 4. Test of complementation on selective agar plates. The plates contained 0.67% Difco yeast nitrogen base, the necessary nucleotides and amino acids except (−Phe) or including (+Phe) phenylalanine, and 2% galactose (Galactose) or 3% glycerol plus 3% ethanol (Glycerol). The permissive yeast host strain 351m was derived from the mutant 351m::1F7* by loss of the antibody-bearing plasmid. Transformation of 351m with plasmids pABZ260 and pABZ265 gave strains 351m::1F7 and 351m::6D4, respectively, where 1F7 refers to the chorismate mutase antibody and 6D4 is an unrelated catalytic antibody with esterolytic activity. Transformation of YT-4Ca and 351m with the plasmid recovered from 351m::1F7* yielded strains YT4Ca::1F7* and 351m::1F7*^{re}, respectively. The plate in *C* was incubated at 25°C for 4 days and stored at 4°C before photography, while the plates in *A* and *B* were incubated at 25°C for 10 days.

sion plasmid pABZ260 (351m::1F7), are able to grow under auxotrophic conditions (Fig. 4*A*). In contrast, transformation of yeast cells with the control plasmid, pABZ265 (Fig. 2), which carries the Fab coding sequence for the unrelated esterolytic antibody 6D4 (5, 6), does not suppress the *aro7* mutation (351m::6D4; Fig. 4*A*). Clearly, expression of large amounts of immunoglobulin protein within the cell is not sufficient for complementation; an antibody with the correct active site and chemical reactivity is required.

It is important to note that the chemical efficiency of the chorismate mutase antibody has not been increased as a result of the mutagenesis experiments: transformation of the parent YT-4Ca strain with plasmid recovered from 351m::1F7* does not produce cells capable of growth under selective conditions (YT-4Ca::1F7*; Fig. 4*A*). Rather, 351m is a permissive host strain. The nature of the genomic mutation(s) that facilitates complementation by the catalytic antibody remains to be investigated and may reflect either improved intracellular expression and/or stability of the immunoglobulin. The slight growth advantage observed for 351m compared to the unmutagenized starting strain YT-4Ca (Fig. 3) is also consistent with an elevated intracellular concentration of chorismate and hence a higher rate for the spontaneous thermal rearrangement, in which case the antibody would only have to supply modest additional activity to the cell to reach the threshold level needed for growth. Additional improvements in expression or efficiency of 1F7 are expected to increase the selective advantage of the antibody-containing cells still further. Indeed, the growth requirement that has been established for antibody activity can now be exploited to evaluate genetic changes in the immunoglobulin molecule itself. Through subsequent rounds of random mutagenesis and genetic selection, for example, it may be possible to augment 1F7's catalytic potency directly (17). The use of biological selection pressure in this way to direct the evolution of immunoglobulin catalysts would have great significance, given that first-generation catalytic antibodies are typically orders of magnitude less efficient than naturally occurring enzymes.

In sum, we have shown that a catalytic antibody can function *in vivo* to complement a defective biosynthetic pathway in a permissive strain of the yeast *S. cerevisiae*. These experiments thus establish the feasibility of directing eukaryotic cells to produce man-made catalysts not normally found in their evolutionary repertoire. In principle, entirely new catalytic functions can now be engineered into such cells, since tailored immunoglobulin catalysts can be prepared for a wide range of chemical reactions, including transformations without known biological counterparts (18). We anticipate that expression of functional antibody catalysts in living organisms will provide exciting opportunities for regulating cellular function, altering cellular metabolism, and destroying toxins *in vivo*.

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