Expression of an Orotate Decarboxylating Catalytic Antibody Confers 5-Fluoroorotate Sensitivity to a Pyrimidine Auxotrophic *Escherichia coli*: An Example of Intracellular Prodrug Activation

Jeffrey A. Smiley and Stephen J. Benkovic*

Department of Chemistry, The Pennsylvania State University University Park, Pennsylvania 16802

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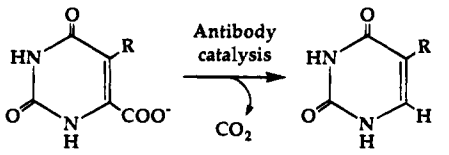
The potential for catalytic antibodies to serve as prodrug activators was recognized early in the development of these artificial catalysts.[1] The ability to create a unique catalyst for which there is no naturally occurring enzyme counterpart would allow an additional degree of specificity in the process of prodrug design, delivery, and conversion to the active drug.[2] Catalytic antibodies are attractive candidates in antibody conjugate technology, where a specifically designed catalyst is linked to a monoclonal antibody which recognizes a target cell surface marker, thus bringing the prodrug catalyst into direct proximity with the target cell.[3]

Using standard monoclonal antibody techniques, various groups have successfully isolated antibodies which convert a prodrug substrate into a toxic product, with a catalytic efficiency sufficient to produce a concentration of product which will slow the growth of bacteria in microbiological assays.[4] We now report development of a system for bacterial expression of single-chain catalytic antibodies in which the recombinant antibody catalyzes formation of a toxic compound within the cell, leading to the suppression of growth of the host cell in which the antibody is produced.

Previously, recombinant antibodies capable of catalyzing the decarboxylation of orotate to yield uracil (Scheme 1, R = H) were produced in a pyrimidine auxotrophic (*pyrF*$^-$) strain of *Escherichia coli* and shown to provide a growth advantage to this strain on pyrimidine-free media.[5] *pyrF*$^-$ strains lack OMP decarboxylase (ODCase) activity[6] and are thus resistant to 5-fluoroorotic acid (5-FOA). In these auxotrophs, 5-FOA cannot be converted to toxic metabolites such as 5-fluoro-2′-deoxyuridine 5′-monophosphate, a potent thymidylate synthase inhibitor,[7] or fluorinated nucleic acids. We examined the ability of orotate decarboxylating antibodies, selected by auxotroph complementation, to confer 5-FOA sensitivity to bacterial strain JFS116 as an assessment of these antibodies' abilities to convert 5-FOA to 5-fluorouracil (5-FU) *in vivo* (Scheme 1, R = F).

Six antibody-encoding plasmids selected in the original complementation screen as functional antibody fragments (Fabs) were converted to single-chain form by the same process as described for antibody SCA-8.[5] To compare the growth of cells and assess the ability of antibodies to produce 5-FU from 5-FOA, cultures of antibody-producing JFS116 and controls were initially raised separately in Luria broth[8] containing 0.2% glucose, 12.5 mg L^{-1} tetracycline, 50 mg L^{-1} kanamycin, and, for plasmid-bearing cells, 30 mg L^{-1} chloramphenicol. Cells were then transferred to minimal media as in the procedure for complementation[5] and plated on minimal media agar plates[5] with 0.3 mM isopropyl β-D-thiogalactopyranoside (IPTG) for induction, 0.5 mg L^{-1} uracil, and variable concentrations of 5-FOA. (This concentration of uracil in the media allows slow growth of the auxotrophs and observation of growth inhibition with 5-FOA.)

Scheme 1



Plasmid pSC-8, which conferred noticeable growth advantage to the auxotroph on uracil-free media,[5] also confers sensitivity to the same strain when grown on 2.9 mM 5-FOA, as determined from the sizes of bacterial colonies arising on growth media with varying components. The sizes of colonies within a given experimental or control group were fairly consistent when well-separated colonies at the end of the inoculating streak were measured, as was observed in the original complementation experiment.[5] Cells with no plasmid, pSCL (single-chain antibody cloning vector[5]), or pSC-43C9 (plasmid encoding an unrelated single-chain antibody) produce colonies of roughly similar sizes in the presence or absence of 5-FOA (Figure 1). Cells carrying pSC-8 yield smaller colonies in the presence of 5-FOA, about 60% the size of control colonies. Plasmid pSC-8 conferred the most sensitivity of the six single-chain antibody-encoding plasmids which were derived from the Fab-encoding versions.

The sensitivity of JFS116 to 5-FU, the product of the antibody-catalyzed decarboxylation, was assayed by measurement of the colony sizes in the presence of varying concentrations of this toxic anabolite. At a concentration of 3 μM 5-FU, colonies of JFS116 with no plasmid grew to about 40% the size of colonies grown in the absence of 5-FU (data not shown). The single-chain antibody SCA-8, encoded by pSC-8, is apparently producing an effective concentration of 5-FU *in vivo* in the micromolar range.

Soluble single-chain antibody was produced from plasmid p4.1.8,[5] using a T3 promoter system.[9] To demonstrate catalytic decarboxylation of 5-FOA *in vitro*, 5 μg of purified SCA-8 was incubated with 5 mM 5-FOA, in a 290-μL reaction mixture containing 20 mM MOPS (pH 7.0) at 37 °C. Aliquots of the reaction were withdrawn at several intervals, and the reactions were quenched by the addition of 1 vol of acetone. After protein was removed by precipitation and centrifugation, 5-FOA and product 5-FU were present in the supernatant. Substrate and product were separated by thin-layer chromatography using silica gel plates and a 9:1 mixture of CH_2Cl_2 and methanol. The product (R_f = 0.4) was easily separated from the substrate (R_f = 0) and quantitated by UV shadowing and comparison to standards. In 36 h, 5 μg of purified SCA-8 produced approximately 0.2 μg of 5-FU; the activity of 1×10^{-4} nmol min^{-1}

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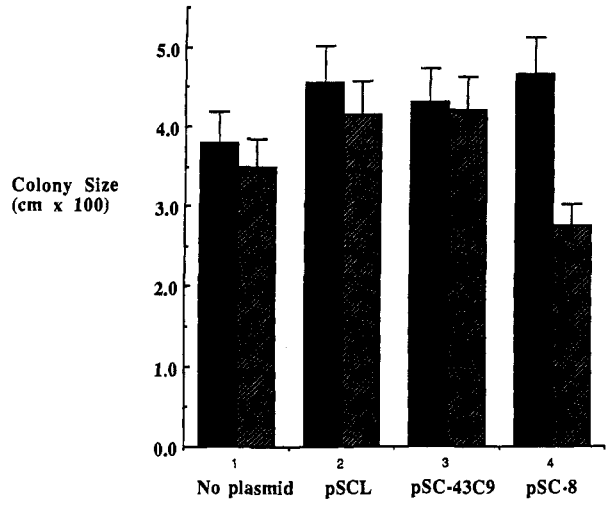

Figure 1. Sizes of colonies of *E. coli* strain JFS116 (*pyrF*$^-$ mutant) carrying various antibody-encoding or control plasmids, grown on media with (hatched bars) or without (solid bars) 2.9 mM 5-FOA. Colonies were visualized and photographed using light microscopy as detailed in ref 5. Sample size was 5−15 clearly separated colonies; the degree of constant size among colonies within a sample set was similar to that oin the colonies pictured in ref 5.

μg^{-1} compares with the value of 1.0×10^{-5} nmol min^{-1} μg^{-1} measured for orotate at 15 μM using a $^{14}CO_2$ displacement assay.[5] Inhibition of 5-FOA decarboxylation by this single-chain antibody was complete in the presence of 0.5 mM 2,4-dihydroxyquinoline (2,4-DHQ), the hapten against which the immune response was originally generated. Using a TLC solvent of ethyl acetate−hexane−methanol (6:3:1), 5-FU (R_f = 0.5) was separable from 2,4-DHQ (R_f = 0.3); no trace of product was detectable in inhibited antibody assays.

The *pyrF*$^-$ mutation in JFS116 results in the accumulation of orotate from the *de novo* pyrimidine pathway; pyrimidine mutants can accumulate orotate to concentrations of 2 mM or greater.[10] This endogenous metabolic intermediate competes at the antibody catalytic site with the added 5-FOA in these sensitivity experiments, and the production of 5-FU is probably slowed by the presence of orotate. In order to eliminate the orotate from the host cells, leaving the single-chain antibody free to catalyze the decarboxylation of only 5-FOA and perhaps develop a more sensitive screen for *in vivo* activity, we constructed a *pyrF*$^-$ strain, which lacks aspartate transcarbamylase (*pyrB*$^-$) and thus does not produce orotate.

Strain YA289 (*pyrB*$^-$) was obtained from the *E. coli* Genetic Stock Center (New Haven, CT). This strain was made *pyrF*$^-$ by a P1 phage transduction,[11] in which the infecting phage was raised on JFS116. The transposable element Tn5, conferring kanamycin resistance, maintains the *pyrF* auxotrophy in JFS116,

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(11) Wild-type P1 phage and protocols for transduction were generously provided by Dr. R. D. Porter, Department of Biochemistry and Molecular Biology, Penn State University. We are grateful for his assistance.

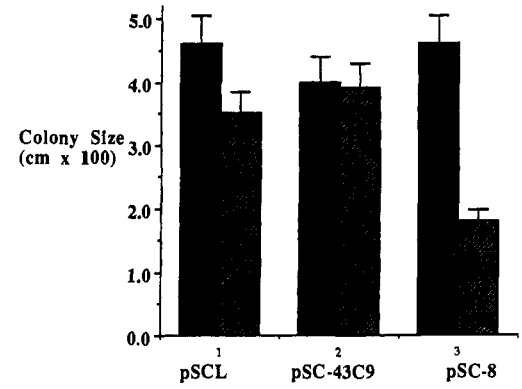

Figure 2. Sizes of colonies of *E. coli* strain PBF1 (*pyrB*$^-$*pyrF*$^-$ double mutant) carrying various antibody-encoding or control plasmids, as in Figure 1.

and a subpopulation of the P1 phage grown on this host acquired the Tn5 marker. Infection of YA289 with this phage population and plating on kanamycin media yielded colonies which were subsequently identified as kan^R, *pyrB*$^-$, and *pyrF*$^-$. This strain, termed PBF1, was transformed with the antibody-encoding and control plasmids for measurement of sensitivity to 5-FOA.

Colonies of PBF1 carrying pSC-8 on media with 5-FOA were again significantly smaller than control colonies. The affected colonies were diminshed to about 40% of the controls, an effect somewhat greater than that seen with JFS116 (Figure 2). The enhanced sensitivity of this strain when producing the single-chain antibody SCA-8 is likely due to the absence of orotate, which would compete for the antibody catalytic site. As an additional control, the sensitivity of PBF1 to 5-FOA with and without the decarboxylating antibody was examined in the presence of the hapten 2,4-DHQ. In the presence of 0.5 mM hapten, the sensitivity of PBF1 expressing the decarboxylating antibody was eliminated; colony sizes in the experimental and control groups were equal.

The utility of catalytic antibodies as therapeutic agents for prodrug activation or detoxification is currently restricted by a number of factors; notable among these factors is their poor intracellular expression in bacterial systems. The development of new methods for screening for activity in recombinant catalytic antibodies, in which the growth of the host is altered by the product of antibody catalysis, should facilitate future efforts to identify antibodies with increased expression levels and/or activity. Mutagenesis and other genetic engineering techniques, in combination with this type of screen, should make possible the rapid identification of efficient catalysts of biologically relevant reactions by virtue of the antibodies' abilities to more completely diminish or enhance growth of the host cell.

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