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Why Increasing Enzyme Abundance Does Not Necessarily Increase Product Formation: A Constraint-Based Theory of Proteome Allocation, Cofactor Competition, and Flux Redistribution in Metabolic Engineering

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Abstract

Increasing the abundance of a pathway enzyme is one of the most intuitive interventions in metabolic engineering, yet additional protein does not guarantee a proportional increase in product formation. This theoretical analysis examines why the relationship between enzyme abundance and pathway output becomes conditional once an engineered pathway is embedded within a resource-limited cell. Published experimental and computational evidence indicates that increased enzyme expression can alter translation-resource demand, proteome allocation, physiological state, cofactor availability, precursor use, protein interactions, and the operating state of the wider metabolic network. These effects create several biologically distinct routes through which additional enzyme can become catalytically underused, impose opportunity costs elsewhere in the proteome, or redirect flux away from the intended product. The resulting interpretation treats enzyme overexpression as a system-level perturbation whose outcome depends on realized catalytic capacity and on the resources and network states required to sustain it. A constraint-based account is developed in which increased abundance improves product formation primarily when the focal enzyme remains limiting after accounting for substrate access, catalytic state, proteome cost, cofactor supply, competing reactions, and regulatory adaptation. This perspective provides a mechanistic basis for distinguishing productive overexpression from interventions that mainly relocate metabolic limitation and establishes testable criteria for evaluating enzyme-expression strategies without assuming proportional abundance–flux coupling.

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Introduction

Increasing enzyme expression appears to offer a direct means of raising metabolic throughput: more catalyst should provide more reaction capacity and, if that reaction limits pathway operation, increase formation of the desired product. The difficulty is that enzyme abundance is changed inside a cell whose biosynthetic machinery is finite. Synthetic expression modules can compete for shared cellular resources, producing interactions between nominally independent constructs that arise from resource demand itself (Qian *et al*., 2017). Engineering feedback against this coupling can alter circuit behavior, demonstrating that the surrounding resource state is an active determinant of expression-system performance rather than a passive background (Shopera *et al*., 2017).

The same principle becomes important when the expressed protein is a metabolic enzyme. Heterologous genetic systems can impose measurable physiological and transcriptional burdens on *Escherichia coli*, and the magnitude and character of those effects depend on construct architecture and expression demand (Liu *et al*., 2018). Increasing enzyme abundance must therefore be interpreted as two simultaneous interventions: an intended change in catalytic capacity and a change in the allocation of the cellular machinery required to synthesize, fold, maintain, and operate that enzyme. These interventions may reinforce one another when the focal reaction genuinely limits production, but they can also work in opposite directions.

Resource coupling is sufficiently consequential that engineered allocation of translation machinery can reduce interference among co-expressed genes (Darlington *et al*., 2018). This observation is conceptually important for metabolic engineering because it shows that an observed response to increased protein expression can partly reflect competition for expression resources rather than only the biochemical action of the protein being expressed. A pathway enzyme whose cellular concentration doubles has therefore undergone a clearly defined molecular change, but the consequences of that change for pathway flux remain conditional.



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Direct pathway-engineering evidence further weakens the assumption that maximal expression is equivalent to maximal productivity. Experimental exploration of enzyme-expression configurations in an isobutanol pathway demonstrated that pathway performance depends on how enzyme expression is distributed and that excessive pathway protein synthesis can itself become limiting (Ghosh *et al*., 2019). Dynamic metabolic-control research likewise treats growth, pathway activity, and production as competing or temporally varying objectives that may require regulated expression rather than permanently maximal pathway activity (Hartline *et al*., 2021). These findings do not imply that overexpression is generally ineffective. They identify the more precise question addressed here: under which conditions does increasing the abundance of a focal enzyme add usable pathway capacity, and under which conditions does it merely relocate the limiting constraint?

Materials and Methods

Enzyme Abundance and the Limits of Flux Proportionality

A proportional relationship between enzyme abundance and product formation requires more than the presence of additional protein. The added enzyme must be catalytically competent, encounter sufficient substrate, operate under an adequate physicochemical and cofactor environment, and occupy a reaction position at which additional capacity can still influence net pathway flux. Even relatively simplified biochemical systems demonstrate this dependence on system state. In *E. coli*-derived cell-free systems, enzyme and metabolite perturbations produce distributed metabolic responses rather than a uniform conversion of added catalytic material into increased output (Miguez *et al*., 2021). Cell-free observations cannot be transferred directly to intact cells, but they establish an important mechanistic point: enzyme amount is only one variable determining effective pathway activity.

Physiological state creates another source of non-proportionality. Quantitative multiomics in glucose-limited *Saccharomyces cerevisiae* showed that functional proteome allocations change systematically with specific growth rate, with distinct protein groups occupying different fractions of the proteome as cellular demand changes (Xia *et al*., 2022). Consequently, the functional meaning of a particular enzyme abundance depends partly on the proteomic state in which it is measured. Identical absolute or relative abundance values need not represent identical spare catalytic capacity in cells operating under different growth rates, nutrient regimes, or competing biosynthetic demands.

The same issue arises in enzyme-constrained metabolic models. Predictions of feasible metabolic states depend on estimates of enzyme turnover capacity, yet turnover numbers are not universally transferable constants for cellular modeling. Integration of enzyme information across conditions improves turnover-number estimation and the predictive performance of proteome-constrained genome-scale models (Wendering *et al*., 2023). This creates an important measurement boundary for overexpression studies. Protein abundance is observable; the fraction of that protein that contributes usable in-cell catalytic capacity is an inferred quantity that depends on catalytic state, metabolic conditions, parameterization, and sometimes on interactions that are poorly captured by bulk protein measurements.

Longer-term adaptation provides a complementary perspective. Proteome partitioning continues to constrain cellular phenotypes during laboratory evolution, indicating that adaptive improvement does not remove the requirement to distribute finite cellular protein among competing functions (Mori *et al*., 2024). Acute enzyme overexpression should not be equated with long-term evolutionary adaptation, but both settings demonstrate that the functional value of protein abundance is embedded in an allocation state. The metabolic neighborhood of an enzyme also matters. Work examining the genetic landscape of a metabolic interaction between dihydrofolate reductase and thymidylate synthase showed that the functional consequences of enzyme variation depend on the surrounding metabolic relationship rather than being fully attributable to the altered enzyme in isolation (Nguyen *et al*., 2024). Thus, even where abundance changes the capacity of one reaction, downstream output can remain conditioned by coupled reactions.

Protein organization provides a further departure from scalar abundance–activity reasoning. Non-consecutive enzymes within the tricarboxylic acid cycle can participate in supramolecular interactions that affect catalytic behavior and the coordination of carbon and nitrogen metabolism (Jasinska *et al*., 2024). This finding does not establish widespread metabolon-like organization for all engineered pathways. It does show that a protein molecule's functional contribution may depend on where it is, what it interacts with, and the state of the surrounding enzyme network. Counting molecules cannot capture those dimensions.

Resource-balance modeling formalizes several of these considerations by representing metabolic activity together with the protein, ribosome, and compartmental resources required to realize it. Application of resource balance analysis to *S. cerevisiae* illustrates how feasible metabolic phenotypes are constrained by allocation of catalytic and biosynthetic machinery (Dinh & Maranas, 2023). Enzyme-constrained extensions of genome-scale models similarly connect reaction feasibility to enzyme capacity rather than permitting flux to vary independently of the protein resources needed to support it (Lu *et al*., 2019). These models remain representations rather than direct observations of causal molecular limits, but their usefulness follows from a biologically grounded point: an increase in enzyme concentration expands one potential capacity while leaving multiple other capacities finite.

The relationship between abundance and product formation can therefore fail at several separable levels. An added enzyme can remain partly unused because substrate supply is insufficient; its effective turnover can differ from the value assumed from another condition; its reaction can cease to be limiting; its metabolic partners can impose a downstream constraint; or its cellular organization can change the meaning of the measured abundance. These mechanisms are not interchangeable, and distinguishing them matters experimentally because each predicts a different response to further overexpression.

The resulting analytical distinctions are summarized in **Table 1**.

Table 1. Conditions that decouple measured enzyme abundance from realized product-forming capacity

Biochemical or cellular condition	What increases experimentally	Why product-forming capacity may increase less than abundance	Diagnostic comparison needed	Interpretation permitted
Focal enzyme remains reaction limiting	Enzyme concentration	Added catalytic sites can still influence reaction throughput	Product/flux response across controlled expression levels	Positive abundance–flux coupling is plausible within the tested state
Substrate delivery becomes limiting	Enzyme concentration	Additional catalytic sites remain insufficiently occupied	Substrate availability or upstream flux versus enzyme level	Limitation has moved upstream
Downstream reaction capacity becomes limiting	Focal enzyme concentration	Increased intermediate formation cannot be propagated proportionally	Intermediate behavior and downstream pathway response	The focal reaction is no longer the dominant control point
Effective catalytic state differs across conditions	Measured protein abundance	Not all measured protein contributes equivalent in-cell catalytic capacity	Condition-specific activity or validated enzyme-capacity estimate	Abundance cannot be used as a direct activity proxy
Enzyme–enzyme or structural context changes	Protein amount or localization	Functional behavior depends on interaction or spatial organization	Interaction/localization perturbation with matched abundance	Network organization contributes to realized capacity
Proteome state changes with growth or environment	Relative or absolute enzyme abundance	The same abundance occupies a different resource context	Matched physiological state and proteome allocation	Abundance should be interpreted conditionally
Model-imposed enzyme capacity changes	Modeled enzyme constraint	Predicted flux depends on parameterization and model structure	Parameter sensitivity and experimental validation	Prediction identifies a testable constraint, not an observed flux

Table 1 separates measured abundance from the conditions required for that abundance to become useful pathway capacity. The distinction also prevents a common interpretive error: a plateau in product formation after further expression does not by itself identify the new limiting mechanism. Substrate scarcity, catalytic-state changes, downstream saturation, proteome competition, or another network effect can generate similar macroscopic behavior. The next section therefore moves from the local abundance–capacity relation to the cellular resources consumed by producing and maintaining additional enzyme.

Proteome Allocation, Cofactor Competition, and Metabolic Opportunity Costs

Protein synthesis occurs within a finite proteome. Resource-allocation models use this constraint to explain why microorganisms can display apparently inefficient substrate-use strategies when faster growth or other cellular functions require allocation of protein capacity elsewhere (Regueira *et al*., 2021). For metabolic engineering, the important implication is that pathway proteins have an opportunity cost. Increasing the fraction of the proteome assigned to a heterologous or amplified pathway necessarily changes the resources available for some combination of central metabolism, transport, translation, maintenance, stress response, and other cellular functions, even when the displaced function is not immediately evident from the measured product pathway.

This allocation is actively regulated. Reconstructing or redirecting transcriptional regulatory networks has therefore been proposed as a means of reallocating cellular resources toward engineered biosynthesis rather than treating pathway expression as independent of host regulation (Ma *et al*., 2022). The consequence for enzyme overexpression is that a gene-level intervention may provoke a regulatory response that alters the rest of the proteome. The resulting production phenotype can reflect both the desired catalytic perturbation and the compensatory allocation response elicited by that perturbation.

Direct metabolic-engineering evidence illustrates the scale such redistribution can reach. Engineering branched-chain acetate-ester production in *E. coli* required substantial reallocation of proteomic resources toward the production system (Seo *et al*., 2022). The exact reported proteome fractions are pathway-specific and should not be generalized to unrelated products. Their broader significance is that high-performing production phenotypes may occupy markedly different proteomic states from their hosts' unengineered states. A strategy that raises one pathway enzyme while leaving the associated allocation problem unresolved may therefore improve one local capacity while degrading another function required for sustained product formation.

Expression burden also provides a rationale for distributing pathway tasks. Resource-aware whole-cell modeling of microbial consortia indicates that division of labour can become

advantageous when the expression cost of performing a complex function in one cell becomes sufficiently consequential (Atkinson *et al*., 2022). Such modeling does not establish a universal threshold at which pathway division becomes preferable. It nevertheless illustrates an alternative response to the same constraint: when enzyme expression itself consumes limiting resources, increasing expression further is not the only available route to increasing effective pathway capacity.

Cofactors constitute a distinct resource class. Cellular reactions frequently require reducing equivalents or other reusable molecular cofactors whose availability is governed by synthesis, regeneration, and competing consumption. Analyses of reducing-power provision in synthetic-cell systems emphasize that supplying NAD(H) or NADP(H) must be designed explicitly when constructing sustained enzymatic reaction systems (Partipilo *et al*., 2023). Living cells possess much richer regulatory and regenerative capabilities, but they also contain many reactions competing for those pools. An enzyme can therefore be abundant while remaining functionally limited by the rate at which its required cofactor is supplied or regenerated.

It is important not to compress these effects into a single undefined concept of "metabolic burden." Reviews of heterologous protein overexpression in *E. coli* distinguish multiple sources of stress, including demand on transcriptional and translational machinery and broader physiological consequences of excessive protein production (Snoeck *et al*., 2024). These mechanisms predict different interventions. A ribosome-limited state should respond differently from a cofactor-limited state, and both differ from a condition in which excessive intermediate accumulation or product toxicity dominates.

Even transcript and protein measurements can obscure this allocation problem if interpreted too directly. Quantitative analyses linking transcriptome organization to proteome allocation indicate that the mapping between RNA abundance and proteomic investment has substantial modular and regulatory structure (Patel *et al*., 2024). Accordingly, increases in transcript abundance do not establish proportional increases in enzyme abundance, and increases in enzyme abundance do not establish proportional increases in pathway flux. Each transition in that chain requires its own evidentiary support.

Large-scale characterization of synthetic constructs offers an additional demonstration of finite expression capacity. Measuring the burden imposed by hundreds of BioBrick constructs revealed that synthetic expression can encounter evolutionary and physiological constraints on constructability (Radde *et al*., 2024). These data arise from synthetic genetic systems rather than metabolic-product pathways, so they should not be used to predict metabolite titers directly. They do, however, reinforce the premise that expression demand has host-level consequences that become increasingly relevant as engineered protein production rises.

Cofactor engineering provides perhaps the clearest metabolic example of why more enzyme can fail to resolve a production bottleneck. In engineered cadaverine production, pyridoxal 5′-phosphate supply and its interaction with precursor use required deliberate balancing rather than unrestricted cofactor-pathway activation (Liu *et al*., 2024). This case is specific to PLP-dependent cadaverine biosynthesis, yet it illustrates a general discriminating principle. If the focal enzyme has already become constrained by a necessary cofactor, increasing focal-enzyme abundance should produce a weaker response than increasing or dynamically matching the limiting cofactor supply. Conversely, if cofactor supplementation produces little change while additional active enzyme increases product formation, the evidence weighs against cofactor scarcity as the dominant constraint.

Overexpression should therefore be evaluated as the creation of a new pattern of resource claims, not merely the addition of catalytic sites. **Table 2** organizes these claims according to what is consumed or displaced and the pathway-level consequence expected if that resource becomes limiting.

Table 2. Resource claims created by enzyme overexpression and their predicted consequences for product formation

Resource claim generated by overexpression	Cellular quantity placed under additional demand	Mechanistic route to reduced marginal benefit	Observation that would support this constraint	Competing explanation that must remain separable
Protein-synthesis allocation	Ribosomes, translational capacity, amino-acid/protein-production resources	Added pathway protein displaces synthesis of other required proteins	Increasing target enzyme coincides with loss of capacity elsewhere in the proteome	Direct toxicity of the expressed protein
Proteome occupancy	Finite cellular protein mass and functional-sector allocation	Focal pathway consumes a larger fraction of cellular protein without equivalent network benefit	Product gain diminishes as pathway protein fraction continues to rise	Substrate limitation independent of proteome cost
Regulatory reallocation	Transcriptional and host-response capacity	Host compensation modifies expression of metabolic or maintenance functions	Global expression state changes reproducibly with the overexpression perturbation	Constitutive pathway-specific regulation
Folding and maintenance demand	Protein-quality-control and maintenance machinery	High expression increases the cost of producing functional protein	Functional enzyme fraction rises less than total protein abundance	Intrinsic catalytic inactivity

Cofactor consumption	NAD(H), NADP(H), PLP, or another pathway-relevant cofactor	Additional enzyme cannot operate at proportional occupancy because required cofactor is limiting	Cofactor-directed intervention restores responsiveness without further focal-enzyme increase	Upstream substrate shortage
Precursor competition	Shared carbon skeletons or pathway intermediates	Greater focal reaction capacity intensifies competition with essential or alternative sinks	Altering precursor supply changes the benefit of overexpression	Downstream reaction limitation
Stress-response demand	Energy and proteins needed for adaptation or damage control	Expression-induced stress redirects resources from production	Stress markers and productivity change together under matched enzyme abundance	Product or intermediate toxicity
Distributed pathway load	Expression resources concentrated in one cell or module	Joint expression of many pathway functions exceeds a useful single-cell allocation state	Redistributing tasks preserves pathway function while reducing local burden	Population-composition effects unrelated to resource cost

The resource classes in **Table 2** predict different experimental outcomes, which is why the theoretical account should resist treating declining marginal returns from overexpression as a single phenomenon. A cell can accumulate more of the intended enzyme while simultaneously losing another capacity required to turn that enzyme into product. Cofactor availability can become limiting without major translational burden; translational burden can occur while the focal enzyme remains catalytically competent; and regulatory adaptation can alter pathway state without changing either mechanism directly. These distinctions are central to interpreting overexpression as a system-level metabolic intervention.

The interaction of the abundance constraints established in "Enzyme Abundance and the Limits of Flux Proportionality" with the allocation constraints developed in "Proteome Allocation, Cofactor Competition, and Metabolic Opportunity Costs" can now be represented without assuming that any one of them dominates universally.

Figure 1 illustrates the proteome, cofactor, and network constraints on product flux.

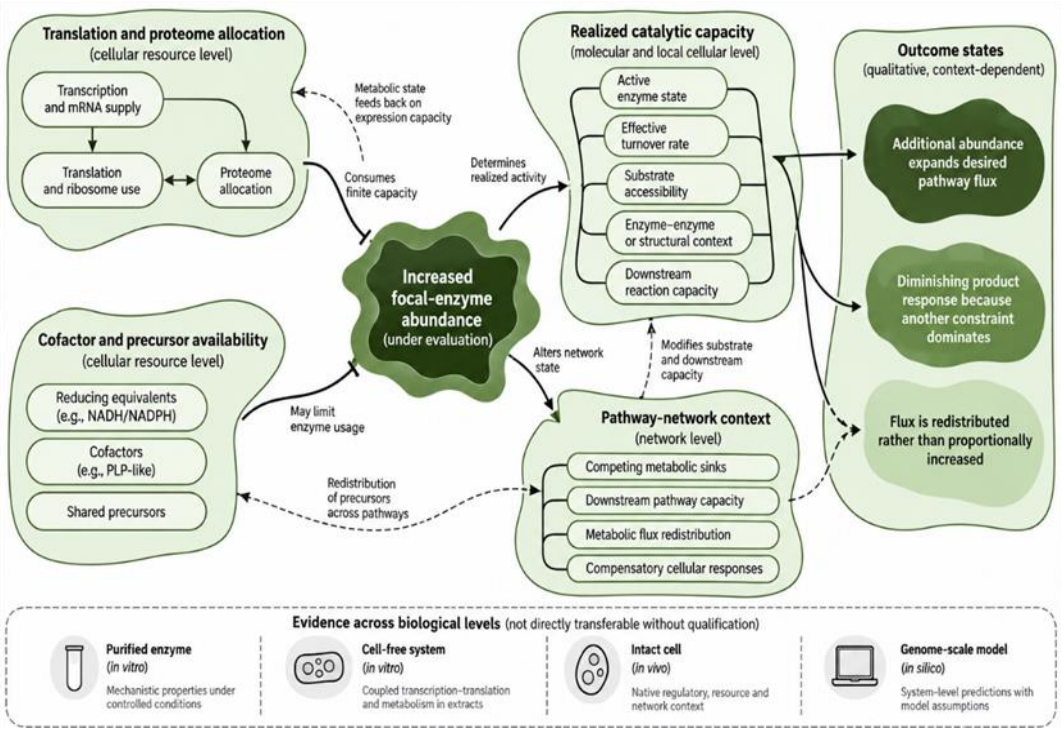


Figure 1. Where Added Enzyme Becomes Nonproductive: Proteome, Cofactor, and Network Constraints on Product Flux

Constraint-Induced Flux Redistribution in Engineered Pathways

Once enzyme abundance, catalytic capacity, and resource allocation are separated conceptually, the central problem becomes one of network state. An engineered cell does not respond to increased enzyme expression by changing only the intended reaction. Altering one reaction capacity can redistribute substrate use, proteome investment, cofactor demand, and competing fluxes. Computational analysis of two-stage production strategies illustrates this principle: metabolic states favorable for biomass formation can differ from those favoring chemical production, making temporal reconfiguration preferable to maintaining a single expression state throughout cultivation (Raj *et al*., 2020). The implication is not that two-stage operation is universally superior, but that a productive flux state cannot be inferred from enzyme abundance independently of the other constraints defining that state.

Flux and proteome allocation can also change on different timescales. Analysis of bacterial growth transitions indicates that metabolic-flux redistribution and proteome reallocation are cross-regulated but need not adjust synchronously (Yuan *et al*., 2024). A measured increase in enzyme abundance may therefore describe a system still moving between physiological states rather than a new steady-state production regime. For overexpression experiments, sampling time consequently becomes part of causal interpretation: identical protein concentrations measured during different phases of adaptation may coexist with different flux distributions.

Spatial organization offers another route by which pathway performance can change without proportional changes in enzyme quantity. Recruitment of sequential pathway enzymes into synthetic protein compartments has been shown to improve desired metabolic flux in engineered *E. coli* systems (Wan *et al*., 2024). Such findings indicate that local substrate transfer and pathway organization can modify the functional return from a given amount of enzyme. They do not imply that compartmentalization is advantageous for every pathway; the relevant conclusion is that catalytic abundance and catalytic arrangement are experimentally separable variables.

Dynamic regulation produces a related effect through time rather than space. A transcriptional repressor-based inverter used for tryptophan-derived biosynthesis altered pathway operation by making expression conditional on cellular state (Gong *et al*., 2024). Intracellular compartment engineering likewise provides mechanisms for concentrating enzymes and substrates, separating competing reactions, or containing intermediates (Wang *et al*., 2023). Together, these approaches demonstrate that pathway flux can be redirected by changing when and where catalytic capacity is deployed rather than only by increasing how much enzyme is present.

Redistribution can occur above the single-cell level as well. Optogenetic control of microbial-consortium composition enabled chemical production to be tuned by changing the relative populations carrying different metabolic functions (Lalwani *et al*., 2021). This population-scale strategy should not be equated with intracellular flux redistribution, but both exemplify the same systems principle: distributing pathway demand differently can alter productive capacity without requiring maximal expression of all pathway components in one cellular state. Functional decomposition of metabolism further shows that metabolic functions carry distinct flux and protein costs, allowing pathway behavior to be interpreted as allocation among competing cellular functions rather than as a collection of independent reactions (Mori *et al*., 2023).

These observations support a constraint-based interpretation of metabolic-engineering interventions, summarized in **Table 3**.

Table 3. Constraint-based interpretation of enzyme-overexpression interventions

Observed response after increasing enzyme abundance	Mechanistically compatible interpretation	Competing interpretation requiring exclusion	Most informative next perturbation	Claim boundary
Product increases approximately with enzyme abundance	Focal reaction remains limiting within the tested range	Concurrent change in substrate supply or regulation	Hold upstream supply constant while varying enzyme abundance	Local proportionality does not establish universal proportionality
Product rises initially and then plateaus	Another pathway capacity becomes dominant	Loss of active enzyme fraction	Perturb downstream capacity, substrate supply, or cofactor availability independently	Plateau does not identify the limiting mechanism by itself
Intermediate accumulates without comparable product increase	Downstream conversion or product-forming step is constrained	Transport or measurement artifact	Modify downstream activity while retaining comparable focal-enzyme abundance	Intermediate accumulation is not equivalent to productive flux
Product decreases at high expression	Resource burden, precursor diversion, cofactor competition, or maladaptive redistribution	Direct enzyme or product toxicity	Reduce expression burden or relieve the suspected shared resource independently	Negative response does not prove one specific burden mechanism

Similar enzyme abundance produces different outputs across conditions	Physiological state or pathway context changes realized capacity	Analytical variation in protein measurement	Match growth state, substrate regime, and sampling time	Abundance must be interpreted conditionally
Spatial or temporal control outperforms constitutive overexpression	Organization or timing is limiting effective pathway use	Different total expression exposure	Compare matched total expression under distinct spatial or temporal designs	Control architecture is pathway specific

The theoretical consequence is that an overexpression intervention should be interpreted by its redistribution signature: which resource claims intensify, which alternative fluxes change, and which previously hidden limitation becomes dominant.

The interaction among these states is summarized in **Figure 2**.

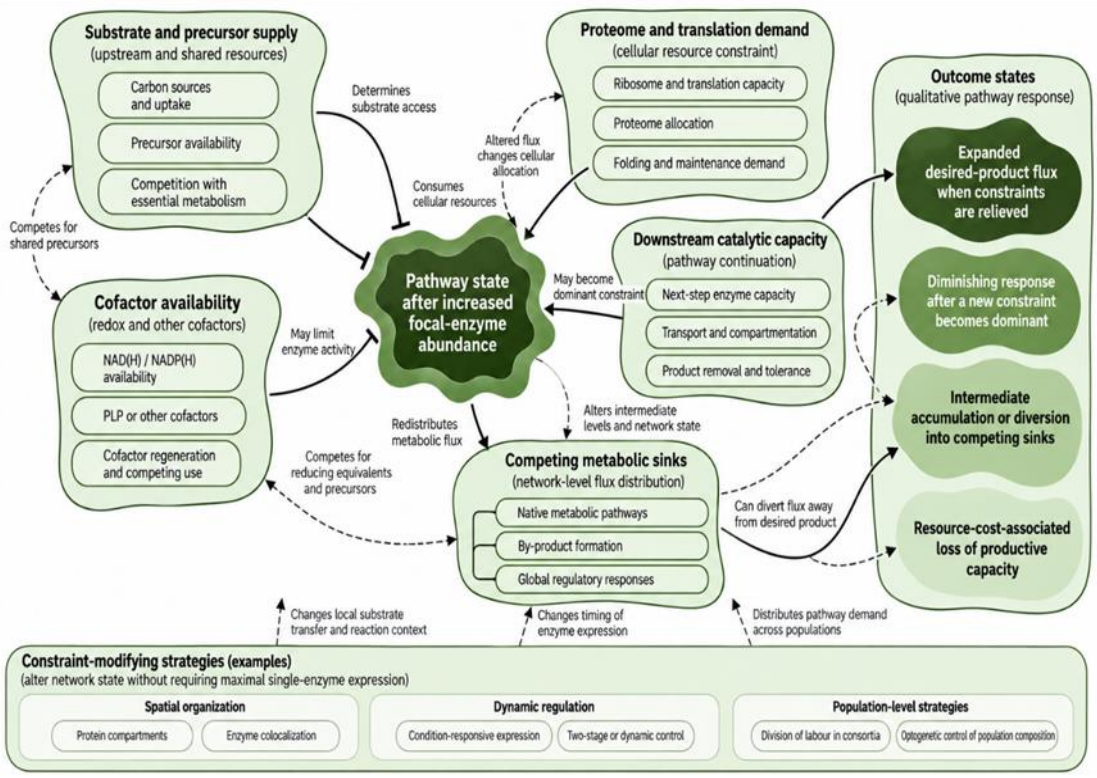


Figure 2. Network States Following Targeted Enzyme Overexpression under Competing Cellular Constraints

A Theoretical Framework for Evaluating Enzyme Overexpression Strategies

The evidence supports evaluating overexpression through a sequence of mechanistic questions rather than through enzyme abundance alone. First, the intervention must be resource feasible: the cell must possess sufficient expression, proteome, precursor, and cofactor capacity to support added enzyme without disabling functions required for production. Resource-allocation constraints materially alter metabolic-model predictions, demonstrating that feasible flux states depend on explicit cellular capacities (Kim *et al*., 2022).

Second, the intervention should be evaluated as a potentially dynamic control problem. A feedback optimizer can, in theory, adjust regulatory variables toward a desired performance state rather than fixing expression at an arbitrarily high level (Gyorgy *et al*., 2023). For metabolic engineering, this implies a discriminating comparison between constitutive overexpression and state-responsive expression under otherwise matched conditions. If dynamic modulation increases productivity without increasing total enzyme exposure, the limiting problem was not simply insufficient enzyme abundance.

Third, computational design and biological validation must remain distinct. Artificial-intelligence methods can assist enzyme and pathway design, identify candidate interventions, and prioritize combinations (Jang *et al*., 2022), but predicted capacity does not establish in-cell feasibility. Any proposed overexpression strategy must therefore survive measurement of actual enzyme abundance,

pathway response, host physiology, and relevant competing constraints.

Finally, host growth and synthetic expression should be treated as coupled components of one system. Resource-aware whole-cell modeling shows how gene-circuit behavior changes when ribosome availability, cellular growth, and synthetic demand are represented together (Sechkar *et al*., 2024). The theoretical account advanced here extends that logic to metabolic engineering: the effect of adding enzyme depends on whether the resulting network state still supplies the resources that make the enzyme useful.

The resulting framework yields three qualitatively different interpretations of an overexpression experiment. A capacity-expanding intervention occurs when added active enzyme remains limiting and increases desired flux without introducing a stronger counterconstraint. A constraint-relocating intervention occurs when added enzyme removes one bottleneck but reveals another, generating diminishing returns. A constraint-amplifying intervention occurs when the resource or network consequences of additional expression offset or reverse the intended catalytic benefit. These are proposed mechanistic classes, not measured thresholds, and their value lies in the different perturbations required to distinguish them.

Results and Discussion

Model Boundaries and Implications for Metabolic Engineering

The practical implication is not that enzyme overexpression should be avoided. It is that successful overexpression is a conditional biological result rather than an engineering default. Metabolite-responsive biosensors provide one route for measuring and controlling pathway state dynamically (Teng *et al*., 2022), allowing expression to respond to intracellular conditions instead of remaining fixed. Their outputs still require independent interpretation because sensor activity is not itself synonymous with metabolic flux or final product formation.

Alternative architectures can also relieve constraints that cannot be solved efficiently by increasing single-cell expression. Dynamic division of labour, for example, can distribute burdensome pathway functions across changing microbial populations (Hamrick *et al*., 2024). Such systems introduce their own population-level constraints, but they illustrate how an unsuccessful overexpression strategy may identify a structural limitation rather than merely an insufficient expression level.

Several observations would restrict the proposed account. If product formation remained proportional to focal-enzyme abundance across substantial perturbations while proteome, cofactor, precursor, downstream-capacity, and competing-flux measures remained unchanged, a resource-redistribution explanation would add little. Likewise, if independent relief of an alleged cofactor or proteome constraint failed to restore responsiveness, that mechanism should be rejected rather than retained as an untestable explanation. Condition-specific turnover estimates (Wendering *et al*., 2023), resource-balance constraints (Dinh & Maranas, 2023), heterogeneous expression burden (Mori *et al*., 2024), and dynamic flux–proteome adjustment (Yuan *et al*., 2024) should therefore be used to formulate discriminating measurements, not to immunize the theory against contradictory evidence.

The framework also has clear transfer limits. Evidence considered here spans cell-free systems, bacterial and yeast cell factories, computational models, and engineered consortia. Relationships demonstrated at one level cannot automatically be promoted to another. The magnitude of proteome reallocation reported for one production pathway (Seo *et al*., 2022), the cofactor logic demonstrated for PLP-dependent cadaverine biosynthesis (Liu *et al*., 2024), and functional protein-cost decompositions developed for specific metabolic systems (Mori *et al*., 2023) should remain source-specific. Host-aware modeling (Sechkar *et al*., 2024) can organize such information, but experimental validation remains necessary.

The strongest use of the theory is therefore diagnostic. When overexpression succeeds, the framework asks which constraint remained unsaturated. When it plateaus, it asks which capacity became dominant. When output declines, it asks whether the intervention created a resource, cofactor, regulatory, or competing-flux penalty. This shifts metabolic-engineering interpretation from "how much enzyme is present?" toward "what network state does that amount of enzyme create?"

Conclusion

Increasing enzyme abundance can raise product formation when the focal reaction remains a meaningful limitation and the cellular system can support the additional catalytic capacity. The relationship ceases to be reliably proportional when substrate delivery, downstream capacity, enzyme state, proteome allocation, cofactor supply, precursor competition, or network adaptation becomes dominant.

The central theoretical implication is that enzyme overexpression is simultaneously a catalytic intervention and a redistribution of cellular resources. Its success therefore depends on the network state produced by the intervention, not on abundance alone. Productive engineering should distinguish added protein from usable catalytic capacity, identify the resources consumed in creating that capacity, and determine whether flux is redirected toward the intended product or toward competing cellular demands.

This constraint-based interpretation makes overexpression strategies experimentally discriminable. Failure of additional expression to increase product formation should trigger tests of alternative limiting capacities rather than repeated escalation of enzyme abundance. Conversely, restoration of responsiveness after relieving a predicted constraint provides evidence for the corresponding mechanism. Enzyme abundance is thus best treated as one controllable variable within a coupled metabolic system whose proteome, cofactors, precursors, regulatory state, and pathway topology jointly determine production.

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