CHAPTER FOUR

Chemical quenching and identification of intermediates in flavoenzyme-catalyzed reactions

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Contents

1. Introduction 90
2. Practical considerations 95
   2.1 Selection of the chemical quencher and reaction conditions 95
   2.2 Detection and analysis of quencher-modified reaction intermediates 97
3. Case study: Flavin-dependent thymidylate synthase 98
   3.1 Chemical quenching protocol 100
   3.2 Analyses of the quencher-modified intermediates 105
   3.3 Buffers and reagents 105
   3.4 Mechanistic insights from the chemical quench 108
4. Concluding remarks 110
Acknowledgments 111
References 111

Abstract

Chemical quenching offers a complementary approach to studying the mechanism of a flavoenzyme, supplementing the information learned from spectroscopic, structural, and computational methods. Generally, in a chemical quench experiment, an enzymatic turnover is quickly stopped at various stages with a chemical agent, and the individual reaction mixtures at each time point are analyzed for the reactants, products and any intermediates. The order by which bonds are made and broken in the reaction is indicated by the identities of the captured intermediates, and the rates of individual steps in the mechanism are determined from the amounts of various chemical species at different time points. This chapter outlines general considerations in selecting a chemical quencher of a particular enzyme-catalyzed reaction and methods for analyzing captured reaction intermediates, with a focus on flavoenzymes. The investigation of flavin-dependent thymidylate synthase is used as a case study to illustrate the concepts and workflow of quenching, isolating, and characterizing quencher-modified reaction intermediates and drawing mechanistic conclusions from the identities of these molecules.
1. Introduction

Flavoenzymes catalyze an impressive variety of chemical transformations, ranging from oxidation-reduction reactions to substitutions of hydrogen atoms with oxygens or halogens to carbon-carbon bond formations, and operate on different substrates, including small molecules, nucleic acids and proteins. To accomplish these chemistries, flavoenzymes have developed an arsenal of mechanisms involving non-covalent reaction intermediates, those covalently bound to the flavin cofactor or the protein, or a combination of thereof. When faced with an uncharacterized flavoenzyme, the question is: where does one begin to dissect the enzyme’s chemical mechanism? Significant insight into the mechanism can be gained by taking advantage of the spectral features unique to a particular flavin intermediate. For example, covalent flavin C4a adducts, such as C4a-peroxy and C4a-hydroxy intermediates in flavin monooxygenases or a C4a-thiol adduct in thiol-disulfide oxidoreductases, are identified by their UV-visible absorbance maxima between 370 and 390 nm (Pimviriyakul, Thotsaporn, Sucharitakul, & Chaiyen, 2017; Ruangchan, Tongsook, Sucharitakul, & Chaiyen, 2011; Sahlman, Lambeir, & Lindskog, 1986; Thorpe & Williams, 1976; Yeh et al., 2006). Likewise, charge-transfer (CT) intermediates involving the
isoalloxazine of the flavin display unique spectroscopic properties: electronic interactions between reduced flavin and oxidized substrates give rise to a blue CT band with absorbance in the 550–650 nm range, while interactions between oxidized flavin and reduced ligands produce a green CT band at longer wavelengths (Ghisla & Thorpe, 2004; Mishanina, Yadav, Ballou, & Banerjee, 2015; Williamson, Engel, Mizzer, Thorpe, & Massey, 1982). If such an intermediate exists, one can use stopped-flow techniques to observe it and characterize the kinetics of its accumulation and decay during the course of the reaction, from which the timing of chemical events involving the flavin can be deduced. What if, however, none of the reaction intermediates have a unique optical signal, or a specific optical signal cannot be readily assigned to a particular intermediate? And how does one probe chemical steps that do not involve the flavin? In these cases, chemical quenching of the reaction can be informative.

In a chemical quench setup (i.e., a quench flow), the reaction of interest is initiated by forcing the reactants together into a reaction loop using a rapid mixer, similarly to a stopped-flow instrument. Unlike in a stopped-flow experiment, however, the reaction is then quickly stopped (quenched) at various time points by adding a chemical agent that disrupts the integrity of the enzyme as a whole or of its active site, thereby preventing any further catalysis (Fig. 1). Examples of chemical quenchers include acids or bases; protein denaturing agents; metal chelators, suited for metalloenzyme-catalyzed reactions; thiol alkylating agents, useful for quenching reactions relying on active-site thiols, and others listed in Table 1 (Barman, Bellamy, Gutfreund, Halford, & Lionne, 2006). Once quenched, the mixture in various stages of reaction is collected and analyzed at each time point for the substrates, products and (hopefully) intermediates that are chemically different from either the starting material or the final product and therefore can be purified from the rest of the mixture and characterized. The order by which bonds are made and broken in the reaction is deduced from the identities of the captured intermediates, and the rates of interconversion between various chemical species are determined from their relative amounts at different time points.

An important caveat of chemical quenching is that most commonly used quenchers, such as strong acid or base, are so reactive that they chemically alter true intermediates to produce their modified forms observed in the analysis of the quenched reaction. This is not to say that the quencher-modified intermediates are not informative. On the contrary,
as demonstrated with the case study in this chapter, these modified intermediates serve as valuable reporters on the timing and nature of chemical steps in the mechanism. Milder quenchers, such as organic solvents or denaturing agents, may be tested to avoid chemical modification of the intermediates by the quencher (Table 1). Alternatively, one can abandon chemical quenchers altogether and stop the reaction physically instead, e.g., by flash freezing, filtering, or rapidly evaporating the mixture into a mass spectrometer (Table 1). In the end, the choice of quenching method depends on the enzyme, stability of the predicted intermediates and the specific question at hand, e.g., whether or not an intermediate is covalently bound to the enzyme. Ideally, a variety of approaches should be used to obtain complementary information about the mechanism of a particular flavoenzyme.
Table 1 Examples of chemical and physical methods to stop (quench) an enzyme-catalyzed reaction.

<table>
<thead>
<tr>
<th>Reaction quencher</th>
<th>When to use</th>
<th>Quencher examples</th>
<th>Applications^a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chemical</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acid</td>
<td>To study kinetics of product formation</td>
<td>Hydrochloric acid; trichloroacetic acid; perchloric acid</td>
<td>Flavin-dependent thymidylate synthase¶,* (Mishanina et al., 2012); ThyA thymidylate synthase* (Moore, Ahmed, &amp; Dunlap, 1986); flavin-dependent halogenase RebH¶ (Yeh et al., 2006); EPSP synthase* (Anderson, Sikorski, &amp; Johnson, 1988); dopamine β-monooxygenase (Brenner &amp; Klinman, 1989); Ca²⁺-ATPase (Kubo, Suzuki, &amp; Kanazawa, 1990)</td>
</tr>
<tr>
<td>Base</td>
<td>Intermediates are likely unstable in acid</td>
<td>Strong base (sodium hydroxide); mild base (trimethylamine, sodium bicarbonate)</td>
<td>Flavin-dependent thymidylate synthase¶,* (Mishanina et al., 2016); EPSP synthase* (Anderson, Sikorski, Benesi, &amp; Johnson, 1988); enolpyruvyl transferase MurZ* (Brown, Marquardt, Lee, Walsh, &amp; Anderson, 1994)</td>
</tr>
<tr>
<td>Denaturing agent</td>
<td>Intermediates are labile in either acid or base</td>
<td>SDS; urea; thiourea; guanidinium chloride</td>
<td>dTDP-glucose 4,6-dehydratase* (Gross, Hegeman, Vestling, &amp; Frey, 2000); interaction of myosin with actin (Van Dijk, Céline, Barman, &amp; Chaussepied, 2000)</td>
</tr>
<tr>
<td>Reducing agent</td>
<td>Oxidized/electrophilic intermediates (e.g., iminium, keto species)</td>
<td>Sodium borohydride or cyanoborohydride; lithium aluminum hydride</td>
<td>UDP-galactopyranose mutase¶,* (Soltero-Higgin, Carlson, Gruber, &amp; Kiessling, 2004); dTDP-glucose 4,6-dehydratase* (Gross et al., 2000)</td>
</tr>
<tr>
<td>Metal chelator</td>
<td>Metal-dependent reactions</td>
<td>EDTA</td>
<td>DNA polymerases (Bryant, Johnson, &amp; Benkovic, 1983); ATP sulphurylase (Liu, Martin, &amp; Leyh, 1994; Sukal &amp; Leyh, 2001)</td>
</tr>
</tbody>
</table>

^aContinued
Table 1 Examples of chemical and physical methods to stop (quench) an enzyme-catalyzed reaction.—cont’d

<table>
<thead>
<tr>
<th>Reaction quencher</th>
<th>When to use</th>
<th>Quencher examples</th>
<th>Applications*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic solvent</td>
<td>Intermediates are labile</td>
<td>Methanol; acetonitrile; acetone, or a mixture of organic solvents</td>
<td>β-1,4-Galactosyltransferase (Wu, Takayama, Wong, &amp; Siuzdak, 1997); glutathione S-transferase (Ge, Sirich, Beyer, Desaire, &amp; Leary, 2001)</td>
</tr>
<tr>
<td>Alkylating agent</td>
<td>Active-site thiol-catalyzed reactions</td>
<td>N-ethylmaleimide; iodoacetamide</td>
<td>Ribonucleoside diphosphate reductase (Erickson, 2001)</td>
</tr>
<tr>
<td>Competitive inhibitor</td>
<td>Potent competitive inhibitor is known</td>
<td>Methotrexate; 5-fluoro-dUMP</td>
<td>Dihydrofolate reductase (methotrexate) (Williams, Morrison, &amp; Duggleby, 1979); ThyA thymidylate synthase* (5-fluoro-dUMP) (Santi, McHenry, &amp; Sommer, 1974)</td>
</tr>
</tbody>
</table>

**Physical**

<table>
<thead>
<tr>
<th>Reaction quencher</th>
<th>When to use</th>
<th>Quencher examples</th>
<th>Applications*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtration</td>
<td>Membrane proteins, immobilized enzymes</td>
<td>0.45 μm Millipore filter</td>
<td>Ca^{2+}-ATPase (Dupont, 1984)</td>
</tr>
<tr>
<td>Flash freezing</td>
<td>Radical intermediates are hypothesized</td>
<td>Isopentane at −140 °C</td>
<td>Xanthine oxidase¶* (Palmer, Bray, &amp; Beinert, 1964); dopamine β-monoxygenase (Mitchell C Brenner, Murray, &amp; Klinman, 1989); M-ferritin ferroxidase* (Krebs, Edmondson, &amp; Huynh, 2002)</td>
</tr>
<tr>
<td>Evaporation into a mass spectrometer</td>
<td>Intermediates are covalently bound to the enzyme</td>
<td>ESI or MALDI ionization methods</td>
<td>Xylanase* (Zechel, Konermann, Withers, &amp; Douglas, 1998); EPSP synthase* (Paiva, Tilton, Crooks, Huang, &amp; Anderson, 1997); dTDP-glucose 4,6-dehydratase* (Gross et al., 2000)</td>
</tr>
</tbody>
</table>

*Note that the list of applications is not meant to be exhaustive. A few examples of enzymatic systems that benefited from the specific quencher are given as a starting point for the reader. In some cases denoted with a “¶”, a reaction intermediate was isolated as a result of the quench. In others, the quencher was used to follow the kinetics of product formation. Flavoprotein examples are marked with a “¶” symbol.
2. Practical considerations

2.1 Selection of the chemical quencher and reaction conditions

As a part of planning a chemical quench-flow experiment, it is helpful to jot down a few mechanistic possibilities for the reaction in question. This will give an idea of predicted stability of the hypothesized intermediates under different solvent conditions, thus suggesting the most appropriate chemical quencher. A few guidelines for the selection of a quenching agent, with examples, are discussed in the following paragraphs.

Acid and base rapidly halt an enzymatic reaction by drastically changing the pH of the solution. This extreme pH change causes the enzyme to denature and often results in the formation of chemical species that are not part of the normal reaction pathway but are instead quencher-modified versions of the true reaction intermediates. Nonetheless, acid is the most commonly used chemical quencher in rapid-quenching experiments and usually the go-to choice. Several phosphoprotein intermediates have been trapped in acid or base and the choice of acid vs. base depends on the enzymatic residue involved in the covalent bond formation. If the residue is histidine, then base quenching should be employed due to the stability of phosphoryl histidine bond in base and not in acid (Van Etten & Hickey, 1977). Likewise, if the covalent bond to the enzyme is formed via a cysteine residue, then this intermediate can be trapped in base due to the stability of phosphothioketals at high pH conditions (Wanke & Amrhein, 1993). We refer the reader to the extensive compilation of covalent intermediates in enzyme-catalyzed reactions, many of which have been captured by chemical quenching (Allison & Purich, 2002).

If both acid and base are expected to decompose reaction components, then denaturing agents such as urea, guanidine hydrochloride and sodium dodecyl sulfate (SDS) can be employed as quenching reagents. As an example, a reaction catalyzed by dTDP-glucose 4,6-dehydratase was successfully quenched in both acid and base but the analytes were decomposed. However, 6M guanidine hydrochloride not only quenched the enzyme but also preserved the integrity of analytes (Gross et al., 2000).

Reducing agents, such as sodium borohydride or sodium cyanoboro-hydride, are known to reduce highly labile iminium ions to form stable amines. Therefore, these reducing agents can be used as quenching agents when iminium ions are proposed as intermediates. Addition of a reducing
agent will stop the enzyme turnover by reducing the reactive iminium ions to form non-reactive amines. Sodium cyanoborohydride quenching allowed the trapping of an N5-alkylated flavin intermediate in uridine 5′-diphosphate (UDP)-galactopyranose mutase by reducing the iminium ion formed on N5 of flavin to form a non-reactive N5-alkylated flavin derivative (Soltero-Higgin et al., 2004).

When applicable, employing different quenching agents to study the same system will allow trapping of different intermediates, as demonstrated with 5-enolpyruvoylshikimate-3-phosphate synthase (EPSP synthase) quenching studies. Even though acid quenching suggested the formation and decay of an intermediate, the structure of the intermediate was not resolved because it was labile in acid. This tetrahedral intermediate was eventually trapped in neat triethylamine and identified as a true, unmodified reaction intermediate (Anderson, Sikorski, Benesi, & Johnson, 1988). The trapping experiments with flavin-dependent thymidylate synthase (FDTS) discussed later in this chapter also showcase the importance of using different quenchers to trap intermediates that are stable under different chemical conditions.

A quench-flow experiment can be time-intensive, particular if anaerobiosis is required. Before heading to a quench-flow apparatus, therefore, it is useful to confirm that the selected quencher indeed quickly (relative to the observed rate constant) stops the enzymatic reaction. This can be done in a couple of ways. First, if the quencher successfully compromises enzyme integrity, then pre-mixing the enzyme with the quencher prior to addition of the required reaction components should not generate any product. This sample is the most appropriate “blank” measurement for product formation. Second, a reaction can be initiated under steady-state conditions and the quencher added to the reaction after a few seconds, after which aliquots are analyzed for product at various time points. If the quencher works, the amount of product should remain constant over time. Once these preliminary controls confirm that the quencher rapidly stops enzyme turnover and the reactants and products are stable in the quenching solution, then the selected quencher can be used in a quench-flow setup.

Intermediates in an enzymatic reaction are generally short-lived and do not accumulate in large quantities. Rapid quench experiments performed under single-turnover conditions (excess of enzyme over substrates) increase the chances of trapping reaction intermediates by ensuring that all of the substrate is bound by the enzyme and thus will react. To further increase the accumulation of intermediates, quench-flow experiments can be carried out at a reduced temperature by taking advantage of the temperature-dependence of reaction rates. This will allow an intermediate
with a lifetime of microseconds–milliseconds at physiological temperature to accumulate due to its reduced reactivity. However, it is important to note that the reaction pathway at low temperatures might be different from that at the physiological temperature under which the enzyme normally operates. Inhibitors that hinder the subsequent chemical step, thus resulting in an accumulation of covalent intermediates, can also increase the probability of trapping an enzyme-bound intermediate. As an example, a variety of 2-deoxy-2-fluoroglycosides (substrate analogues) were used to successfully trap enzyme bound α-glycosyl intermediates in retaining β-glycosidases (Dan et al., 2000; Wicki, Rose, & Withers, 2002). Finally, mutagenesis of the enzymatic residues predicted to play a role in the chemical mechanism is another tool to trap fleeting reaction intermediates, by either slowing the overall rate of the reaction or blocking chemistry that consumes the intermediate (Lairson et al., 2004; Miller et al., 1990; Recksiek, Selmer, Dierks, Schmidt, & von Figura, 1998).

### 2.2 Detection and analysis of quencher-modified reaction intermediates

Samples collected during a chemical quenching experiment might require additional workup before the analysis for reaction components. With acid (e.g., trichloroacetic acid, HCl) as the quenching agent, the enzyme along with any enzyme-bound intermediates precipitates and can be easily separated from the soluble reaction components by either centrifugation or filtration of the quenched sample. Soluble components can then be analyzed for substrates, products and non-covalently bound intermediates, while the precipitated enzyme may be studied for the presence of covalent intermediates. Base-quenched samples should be neutralized before injection onto an HPLC column to avoid any damage to the column resin. Additionally, unlike in acid, denatured enzymes do not precipitate in base and should be removed from the sample via ultrafiltration. Finally, if the quenched sample contains excessive amounts of salts that may interfere with downstream analysis, e.g., mass spectrometry, desalting or buffer exchange might be required before analysis.

Quenched reaction mixtures typically contain multiple chemical components, not all of which participate in the enzyme-catalyzed reaction. One can separate these components using various chromatographic techniques, such as normal- or reverse-phase liquid chromatography, gas or thin layer chromatography. To ease tracking of the species directly involved in the enzymatic reaction, substrates and/or cofactors labeled with either radioactive or stable heavy isotopes are valuable. These labeled reaction components not only make the reacting compounds stand out of the
background, but also offer the advantage of requiring small amounts of material for detection and analysis, which becomes crucial when the intermediates do not accumulate in large quantities. If such labeled substrates are used, one may keep track of the total “counts” (either radioactive counts or ion counts in a mass spectrum) between different chemical species throughout the time course of the reaction. “Missing” counts at any time point suggest existence of an intermediate bound to the enzyme and may be looked for by either gel electrophoresis or mass spectrometry on the protein fraction of the quenched samples. Beyond its value in detecting and isolating trapped intermediates, isotopic labeling is useful for testing what components of the substrate or cofactor structure are parts of the intermediate. For instance, as discussed in the case study, labeling individual rings of the flavin’s isalloxazine one at a time showed that only two of the rings remain intact in the base-modified intermediate.

Ideally, mass spectrometry and radiolabeling experiments would provide enough information for determination of the structure of quencher-modified intermediates. If not or if sufficient quantity of purified intermediate is easily generated, various NMR experiments can be performed to deduce the chemical structure of the trapped intermediate. Obtaining enough material for an NMR spectrum can be tedious and time consuming but it is possible by collecting multiple quenched samples around the time point of the maximum accumulation of the intermediate and purifying the intermediate out of the crude reaction mixtures.

3. Case study: Flavin-dependent thymidylate synthase

Thymidylate synthase encoded by the thyA gene (TYMS in humans) catalyzes the reductive methylation of uridylate (dUMP, 2′-deoxyuridined-5′-monophosphate) to form thymidylate (dTMP, 2′-deoxythymidine-5′-monophosphate), an essential building block of DNA (Carreras & Santi, 1995). Genomes of several pathogenic bacteria lack thyA (e.g., Helicobacter pylori, Rickettsia species and Bacillus anthracis) and instead rely on the flavin-dependent thymidylate synthase (FDTS) encoded by the thyX gene for thymidylate biosynthesis (Myllykallio et al., 2002). These two thymidylate synthases are different in structure, chemical mechanism and co-factor requirement. With FDTS, (6R)-N⁵,N¹⁰-methylenetetrahydrofolate (CH₂H₄folate) serves as the carbon source, in the form of a methylene, and the non-covalently bound flavin adenine dinucleotide (FAD) prosthetic group catalyzes the oxidation-reduction chemistry to form the methyl group of dTMP (Fig. 2A). The chemical mechanism of thyA thymidylate synthase is
Fig. 2 Reaction and the latest mechanistic proposal for flavin-dependent thymidylate synthase (FDTS). (A) Overall chemical conversion catalyzed by FDTS. In the reductive half-reaction (downward arrow), the oxidized FAD cofactor is reduced to an anionic hydroquinone form of the flavin. The physiological reductant is likely nicotinamide adenine dinucleotide phosphate (NADPH); however, for the purposes of the chemical quench experiment, FAD can be reduced with sodium dithionite. In the oxidative half-reaction (upward arrow), the methylene provided by (6R)-N⁵,N¹⁰-methylenetetrahydrofolate (CH₂H₄folate) is reduced by the flavin into a methyl group of the final dTMP product. (B) Proposed chemical mechanism for FDTS, where flavin relays the methylene from the folate donor to the nucleotide acceptor. The mechanism was based on the chemical trapping of intermediates I₁ and I₂, described in the main text. Inset: stacking of the folate’s pterin, flavin’s isoalloxazine and dUMP in the crystal structure of a ternary FDTS complex, consistent with the proposed path of methylene transfer (PDB ID: 4gta; an unreactive CH₂H₄folate analogue, folinic acid, was used in the structure) (Koehn et al., 2012). Note that the chemical structures in the mechanism have been slightly tilted out of the plane of the page to mimic the view in the crystal structure. The rings of the isoalloxazine core are labeled as “a/b/c” for reference. R = 2′-deoxyribose-5′-phosphate; R’ = (p-aminobenzoyl)glutamate; R” = adenosine-5′-pyrophosphate-ribityl.
extensively studied and well understood, which led to the development of mechanism-based inhibitors used as clinical drugs, such as 5-fluorouracil and raltitrexed (Carreras & Santi, 1995; Finer-Moore, Santi, & Stroud, 2003). The mechanism of FDTS, on the other hand, is not clearly established. This lack of mechanistic knowledge on FDTS hampers the development of specific antibiotics with minimal toxicity to humans.

Chemical trapping of intermediates along the reaction pathway with both acid and base as reaction quenchers resulted in a revised chemical mechanism of FDTS (Fig. 2B). Specifically, the identity of an intermediate trapped in base suggested that the flavin prosthetic group in FDTS transfers the carbon from the folate to the nucleotide substrate, in addition to providing the reducing hydride in the end. Here, we discuss the use of two chemical quenchers, acid and base, in a rapid quench-flow experiment and characterization of trapped intermediates to solve the mechanistic puzzle of FDTS. Chemical quench experiments were carried out at room temperature with FDTS from a hyperthermophilic organism Thermotoga maritima, which normally grows optimally at the physiological temperature 80 °C. Sub-physiological temperature allowed a slower overall reaction and thus increased the magnitude of intermediate accumulation.

3.1 Chemical quenching protocol

3.1.1 Equipment

- KinTek Chemical Quench-Flow (QF) instrument model RQF-3
- Anaerobic glass tonometer for deoxygenating and loading the enzyme onto the QF (Fig. 3A)
- Hamilton gastight syringes
- Hamilton threaded plunger 1700
- Schlenk line (a.k.a. vacuum manifold)
- Round-bottom Schlenk flasks, 200 and 50-mL
- Precision seal rubber septa

3.1.2 Buffers and reagents

- Reaction buffer for quenching experiment: 200 mM Tris-HCl pH 8.0
- Glucose oxidase powder from Aspergillus niger and D-glucose
- [2-14C]dUMP (Moravek Biochemicals)
- CH2H4folate solution: 100 mM stock in the reaction buffer
- Formaldehyde stock: 36.5% by weight
Purified *T. maritima* FDTS: 1 mL of 100 μM (active-site FAD concentration, $\varepsilon_{450} = 11,300 \text{ M}^{-1} \text{ cm}^{-1}$) (Mishanina, Corcoran, & Kohen, 2014)

- Sodium dithionite
- Ultra-high purity argon
- Quenching agent: 1M HCl or 1M NaOH

Fig. 3 Overview of the quench-flow instrument setup. (A) Anaerobic tonometer for enzyme reduction, with a gastight syringe attachment for loading the enzyme into the instrument’s sample line. (B) Loading of the enzyme (or an enzyme-dUMP complex) into the tonometer through the side-arm. (C) Tonometer connected to the vacuum manifold (a.k.a. the Schlenk line) through a rubber hose for degassing. (D) Titration syringe with dithionite inserted into the anaerobic tonometer. (E) Tonometer connected to the sample line B and secured with a clamp. (F) Complete setup ready for sample collection.
3.1.3 Procedure

Note: Fig. 3 provides pictures of a few of the steps described below, and Fig. 4A summarizes the workflow described below. The amount of dithionite required to stoichiometrically reduce FDTS-bound FAD can be determined beforehand by following the FAD UV-visible absorbance spectrum upon gradual addition of dithionite in a spectrophotometer (Fig. 4B).

1. Clean the drive syringes and the reaction loops of the quench-flow system with deionized water.
2. Fill the water bath with deionized water and seal the bottom inlet with a rubber septum (Fig. 3F). Secure the septum with electrical tape.
3. In a 250-mL round-bottom Schlenk flask, deoxygenate 75 mL of reaction buffer containing 10 mM glucose for ~30 mins by exposing the solution to alternating cycles of vacuum and ultra-high purity argon.
4. Add glucose oxidase (50 units/mL final concentration) to the anaerobic buffer with a gastight syringe through the septum of the flask.
5. Rinse the drive syringes (except the quencher syringe) and the reaction loops with the anaerobic buffer containing glucose oxidase, fill the syringes with remaining buffer and allow to incubate for ~6 h to scrub the oxygen in the system.
6. Insert a needle through the rubber septum from step 2 and bubble the water bath with argon.
7. Attach an oil bubbler to the outlet of the water bath to maintain the positive argon pressure (Fig. 3F).
8. Let the argon flow through the water bath while the glucose oxidase from step 5 scrubs the system of oxygen.
9. Repeat step 3 and 4 and set aside the anaerobic reaction buffer.
10. Deoxygenate 3 mL of glucose buffer for ~15 mins in a 50-mL Schlenk flask by exposing the solution to alternating cycles of vacuum and argon.
11. Add formaldehyde (final concentration 30 mM) and glucose oxidase (50 units/mL final concentration) with a gastight syringe through the septum of the flask from step 10 and set aside, kept under the positive pressure of argon to maintain anaerobiosis. This solution is later used for CH$_3$H$_4$folate dilution. Formaldehyde stabilizes the labile methylene on the folate.
12. Assemble the tonometer and load the enzyme (1 mL of 100 μM FDTS) mixed with [2-$^{14}$C]dUMP (30 μM final concentration) through the side arm (Fig. 3A and B).
Fig. 4 Chemical quench of a single-turnover FDTS reaction. (A) Flow diagram of the experimental steps. Refer to Fig. 3 for the photos of steps 1–3. (B) Stoichiometric reduction of the oxidized FDTS-bound FAD (Enz-FAD) with sodium dithionite (step 1 in A), followed by the UV-visible absorbance of the flavin. (C) Reverse-phase HPLC radiograms of a 2 s reaction with [2-14C]dUMP as a substrate, quenched with either strong acid (top) or strong base (bottom). The chemical structures of the quencher-modified intermediates are shown (refer to Figs. 5 and 6 for structure determination experiments). The sphere represents the methylene supplied by the folate substrate. (D) Base-modified intermediate kinetics (square data points) overlaid with acid-modified intermediate data (round data points), globally fitted to a two-intermediate model (solid curves). The sum of the two intermediates is shown as a dotted curve. Each data point results from a radiogram like in (C) (shaded slice represents the 2 s time point).
13. Attach the tonometer to the Schenk line and deoxygenate for ~30 mins (Fig. 3C)
14. Bubble 10mL of reaction buffer in a volumetric flask with ultra-high purity argon for 10 mins.
15. Dissolve dithionite powder (final concentration 5mM) in the deoxygenated buffer from step 14.
16. Load dithionite into the syringe with the threaded plunger.
17. Insert the dithionite syringe into the tonometer and gently secure it with the screw cap on top of the tonometer (Fig. 3D).
18. Reduce the enzyme-bound FAD by adding the appropriate amount of dithionite and check for the color change of the enzyme from yellow to colorless.
19. Remove the needle connected to water bath from step 6.
20. Empty the drive syringes from step 5 and load the syringes on either side with fresh degassed buffer from step 4 and the middle syringe with the quenching reagent (aerobic).
21. Attach the tonometer to the quench-flow instrument, secure it with a clamp (Fig. 3E) and prime the sample line B with enzyme solution.
22. Add CH$_2$H$_4$folate (800μM final concentration) to the anaerobic buffer from step 11.
23. Load CH$_2$H$_4$folate solution into a gastight syringe and connect it to the sample line A of the quench-flow instrument (Fig. 3F).
24. Prime the sample line A with the CH$_2$H$_4$folate solution.
25. Turn on the quench-flow instrument and select the appropriate sample loop according to the time point of interest.
26. Load enzyme and the CH$_2$H$_4$folate solutions into the sample lines (20μL each) and start the reaction.
27. Collect the quenched samples in 1.5-mL Eppendorf tubes and freeze them immediately on dry ice till analysis.

3.1.4 Notes
1. All the glass stopcocks on the tonometer, Schlenk flasks and vacuum manifold need to be well greased before each experiment and care should be taken not to over grease to prevent clogs in the bore. When choosing the grease, select a cryogenic high-vacuum grease (Apiezon N-00025).
2. High vacuum ground glass stopcocks and plugs are numbered to avoid accidental mix-ups. When assembling the tonometer, match the numbers of glass joints for a tight seal.
3. When degassing enzyme solution, do not expose it to vacuum for more than 2s or so, as this will cause the enzyme to bubble up and denature.
4. When inserting the syringe containing dithionite, keep the argon flowing through the tonometer to maintain the anaerobicity.
5. Once the enzyme is stoichiometrically reduced with dithionite, set the tonometer aside for ~5 min to see whether the flavin remains reduced, i.e., colorless. This will ensure the anaerobicity is maintained inside the tonometer.
6. Store CH$_2$H$_4$folate in an amber vial or keep it covered to avoid exposure to light and dilute the CH$_2$H$_4$folate stock solution toward the end of the setup to avoid decomposition. CH$_2$H$_4$folate is known to be stable at a high concentration, hence the choice of 100mM for the stock concentration.
7. Sample loops should be cleaned at least once by doing a blank run with buffer before first use and should be cleaned between time points in the same manner to avoid contamination with the mixture from previous reactions. *Note:* this is in contrast to the vacuum-water-methanol cleaning protocol typically performed between reactions on the KinTek RQF, which would compromise anaerobicity of the system.
8. Keep the quenched samples frozen until ready to analyze to avoid potential decomposition of the trapped species.
9. This experiment is performed under anaerobic conditions. However, the chemical quenching step is not.
10. If the quench-flow instrument is placed in a glove box, then steps 5 through 8 of the Procedure can be skipped.

### 3.2 Analyses of the quencher-modified intermediates

#### 3.2.1 Equipment

- HPLC instrument
- Reverse-phase analytical column (Supelco, Discovery series 250 mm × 4.6 mm)
- Table-top centrifuge
- Radioactivity flow-scintillation counter
- LC-MS instrument

#### 3.3 Buffers and reagents

- HPLC separation buffer: 50 mM potassium phosphate (KH$_2$PO$_4$) pH 6.0 and methanol
- LC-MS eluent: water and acetonitrile, both containing 0.1% formic acid
3.3.1 Notes

1. Centrifuge or filter the thawed quenched samples to remove any denatured enzyme before injection onto the HPLC column, to prevent clogging of the column.

2. Base-quenched samples should be neutralized to pH 7–8 before injecting them onto the HPLC, to avoid damage to the column resin.

3. Mobile phase for HPLC separations is a gradient of 50 mM KH2PO4 pH 6.0 and methanol. LC-MS mobile phase is a gradient of water and acetonitrile, both containing 0.1% formic acid.

4. For NMR experiments, base-modified intermediate was purified out of multiple quenched 1 s reactions by HPLC in phosphate buffer and freeze-dried in a 50-mL Falcon tube (tube 1). The compound was desalted via trituration into methanol: (i) the powder was dissolved in methanol; (ii) the supernatant was transferred into a fresh Falcon tube (tube 2), and (iii) methanol was evaporated by blowing argon over the solution in tube 2. Steps i–iii were then repeated with the remaining salt precipitate in the original tube 1. Desalted compound in tube 2 was dissolved in D2O for NMR analyses.

Initial indirect evidence for accumulation of an intermediate(s) in the FDTS-catalyzed reaction came from the LC-MS analysis of nucleotide substrate (dUMP) and product (dTMP) in acid-quenched reactions with unlabeled reactants (Mishanina et al., 2012). In the analysis, the sum of ion counts between dUMP and dTMP was not conserved during the course of the reaction: the total counts decreased in the 0.5–10 s time window, pointing to a “missing” nucleotide material different from either dUMP or dTMP. Use of radiolabeled reactants afforded direct detection, isolation, and identification of the reaction intermediates, as detailed below.

When acid-quenched samples of the FDTS reaction with [2-14C]dUMP were analyzed by HPLC, a radioactive peak different from both the dUMP substrate and the dTMP product was indeed identified (Fig. 4C, top panel), suggesting that this new species contained the pyrimidine ring of the nucleotide substrate. The fraction of the total radioactivity in this new radioactive peak first increased and then decayed with time, indicative of an intermediate. The maximum accumulation of this new radioactive peak occurred around 2 s and corresponded to ~80% of the total radioactivity (Fig. 4D, round data points). In addition, when [11-14C]CH2H4folate, i.e., radiolabeled at the methylene, was used as the only radioactive species in the reaction, the HPLC chromatogram showed a new peak with the same retention time as the intermediate formed when [2-14C]dUMP was used.
This observation suggested that the trapped intermediate is comprised of both the pyrimidine ring of the nucleotide substrate and the methylene group of CH$_2$H$_4$folate (sphere on the intermediate structure in Fig. 4C), giving insight into the order of events during FDTS catalysis, i.e., that the methylene transfer from CH$_2$H$_4$folate to dUMP occurs prior to the formation of the intermediate trapped in acid. In order to identify the acid-trapped species, a separate quench-flow experiment was carried out with nonradioactive substrates, and several 2s reactions were collected where the intermediate shows maximum accumulation (Fig. 4D). Trapped intermediate was purified by HPLC and analyzed by electrospray ionization mass spectrometry (ESI-MS). The exact mass and atomic composition of the acid-modified species corresponded to the product dTMP plus a hydroxyl group (Fig. 5A), i.e., 5-hydromethyl-dUMP. An LC standard of 5-hydroxymethyl-dUMP was synthesized to confirm the identity of the trapped intermediate as such. Retention time on the LC for both the synthetic standard and the trapped species overlapped (data not shown (Mishanina et al., 2012)) and the MS spectra showed the same mass for the two molecules. In addition, the MS-MS spectra of the 5-hydroxymethyl-dUMP and trapped intermediate displayed the same fragmentation pattern (Fig. 5B) confirming the identity of the trapped species as 5-hydroxymethyl-dUMP.

Base-quenched FDTS reactions with both [2-$^{14}$C]dUMP and [11-$^{14}$C]CH$_2$H$_4$folate showed a radioactive peak with same retention time (Fig. 4C; data with the labeled folate not shown (Mishanina et al., 2016)).

Fig. 5 Mass spectra of the product of acid modification of intermediates I$_1$ and I$_2$. (A) Negative-ion ESI-MS spectrum. (B) Negative-ion ESI-MS-MS spectrum, with the structures corresponding to each fragment shown. These data are identical to the MS of an independently synthesized 5-hydroxymethyl-dUMP standard (data not shown (Mishanina et al., 2012)), confirming the identity of the product of acid modification of intermediates I$_1$ and I$_2$ as 5-hydroxymethyl-dUMP. The sphere represents the methylene supplied by the folate substrate.
This new peak elutes much later than the acid-modified intermediate under the same LC conditions, suggesting that these two intermediates are chemically dissimilar. Nevertheless, both contain the pyrimidine moiety of the nucleotide and the methylene carbon of the CH$_2$H$_4$folate (sphere in the structures in Fig. 4D). The mass of the trapped intermediate was 705 Da (Fig. 6A) and tandem mass spectrum of the trapped intermediate showed a mass fragment of 385 Da (Fig. 6B), which did not account for any folate derivative, buffer or protein components. When [7α,8α-3H]FAD–FDTS was used along with [2-14C]dUMP, the base-modified intermediate contained both 14C and 3H radioactive labels indicating that the trapped species contains both the pyrimidine ring and the dimethylbenzene portion of FAD (Fig. 6C). A trapping experiment with [dioxopyrimidine-13C,15N]FAD reconstituted FDTS showed a 2 Da increase in the mass of the trapped species suggesting only a part of the dioxopyrimidine ring is connected to the trapped intermediate (Fig. 6D). The UV-visible absorbance spectrum of the purified and desalted base-modified intermediate suggested an N5-alkylated flavin (Fig. 6E). The final step in characterization of the base-modified intermediate was obtaining NMR spectra of the intermediate, which corroborated the substrate labeling experiments carried out by using labeled FAD and confirmed the chemical structure in Fig. 6E (data not shown (Mishanina et al., 2016)).

3.4 Mechanistic insights from the chemical quench

Once the structure of a quencher-modified intermediate is fully characterized, one can seek to identify its implications for the chemical mechanism of the enzyme. In the case of FDTS, the unexpected covalent link between the flavin cofactor and the methylenated nucleotide substrate in the base-modified intermediate suggested that the flavin serves as a shuttle, accepting the methylene from the folate (step 1 in Fig. 2B) and passing it on to the pyrimidine substrate (steps 2–3 in Fig. 2B), thus calling for a drastic revision of the previously proposed mechanisms (Conrad, Ortiz-Maldonado, Hoppe, & Palfey, 2014; Koehn et al., 2009, 2012; Mishanina et al., 2014). Here, chemical quench revealed a mechanistic detail other experimental methods missed. The use of different quenchers of the same reaction provided complementary data about the intermediates. Specifically, while the acid converted both intermediates I$_1$ and I$_2$ into a single chemical species (5-hydroxymethyl-dUMP, Fig. 4C), alkaline quench resolved these by
Fig. 6 Characterization of the product of base modification of intermediate I1. (A) High-resolution negative-ion ESI-MS and (B) ESI-MS-MS of the base-modified intermediate. The major site of fragmentation is marked with an arrow. The mass of the molecular ion is consistent with the chemical formula of the shown structure. (C) Reverse-phase HPLC radiogram of a 1 s reaction of [7α,8α-3H]FAD-FDTS with [2-14C]dUMP as a substrate, with the two panels showing signal from 14C-(top) and 3H-(bottom) containing species in the reaction mixture. Importantly, the base-modified intermediate has both 14C and 3H signals associated with it, suggesting that dimethylbenzene of the flavin’s isoalloxazine (ring a in Fig. 2B) is a component of the base-modified intermediate. Carbons 7α and 8α carrying tritiums are labeled. The sphere represents the methylene supplied by the folate substrate. (D) High-resolution negative-ion ESI-MS of the base-modified intermediate isolated from the reaction of [dioxopyrimidine-13C,15N]FAD-FDTS with unlabeled substrates (dioxopyrimidine is the ring c of the flavin’s isoalloxazine core, Fig. 2B). The mass of the molecular ion and its sodium adducts is increased by 2 Da, consistent with retention of carbons 4a and 10a in the base-modified intermediates but not the rest of dioxopyrimidine. The final chemical structure of the base-modified intermediate was determined by a suite of NMR experiments (data not shown (Mishanina et al., 2016)). (E) UV-visible absorbance spectrum of the purified base-modified intermediate. The 328-nm peak is characteristic of an N5-monoalkylated flavin (Soltero-Higgin et al., 2004).
capturing exclusively the earlier intermediate \( I_1 \) (Fig. 4D). Additionally, the presence of the methylene on the trapped intermediates at early time points suggested that the methylene transfer (steps 1–3 in Fig. 2B) occurs quickly (a few seconds at room temperature), while the remaining time of the turnover (tens–hundreds of seconds) is spent on the reduction of the methylene into a methyl by the reduced flavin (step 4 in Fig. 2B).

The identities and timing of the chemically trapped intermediates \( I_1 \) and \( I_2 \) agree with the stopped-flow trace of the enzyme-bound FAD at 420 nm (Conrad et al., 2014). First, a decrease in 420-nm absorbance observed with \( I_1 \) accumulation is consistent with the formation of an N5-monoalkylated flavin adduct, which lacks absorbance at 420 nm (Fig. 6E). Second, an increase in 420-nm absorbance corresponding to flavin oxidation accompanies disappearance of the exocyclic methylene intermediate \( I_2 \) and appearance of the dTMP product (Conrad et al., 2014; Mishanina et al., 2014). Together, chemical quenching and stopped-flow experiments with FDTS provided information on the role of the flavin prosthetic group in catalysis, as well as the sequence and timing of chemical steps.

4. Concluding remarks

Chemical quenching has been used to dissect a wide range of enzyme mechanisms for over 50 years (Barman & Gutfreund, 1964). As we hope the reader took away from this chapter, chemical quenching will continue to provide valuable insights into the mechanisms of flavoenzymes that other methods cannot access. In particular, chemical quench allows determination of the kinetics for substrate consumption, product formation and accumulation and decay of intermediates, along with effects of enzymatic mutations, and flavin and substrate analogues on these kinetics. Additionally, from the identities of the trapped, spectroscopically silent, intermediates one can learn the order by which bonds are broken and new bonds are formed in the reaction, with sometimes-unexpected connectivities appearing in the isolated intermediates. As with any other experimental tool, however, chemical quenching does come with its own set of limitations that should be kept in mind, such as (i) high likelihood that the quencher chemically modifies the intermediates; (ii) information about the surrounding environment of the intermediate in the enzyme’s active site (e.g., ion-pair or charge-transfer interactions) is lost upon the quench, and (iii) analysis of the quenched samples is quite labor intensive as reactions from individual time points have to be analyzed one at a time.
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