

Chemical Mechanism Identification from Frequency Response to Small Temperature Modulation

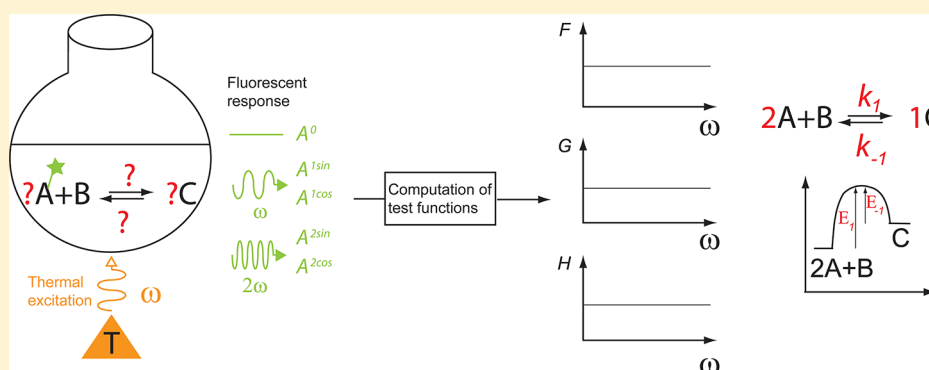
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ABSTRACT: The description of interactions between biochemical species and the elucidation of the corresponding chemical mechanisms encounter an increasing interest both for the comprehension of biological pathways at the molecular scale and for the rationalization of drug design. Relying on powerful experimental tools such as thermal microfluidics and fluorescence detection, we propose a methodology to determine the chemical mechanism of a reaction without fitting parameters. A mechanism consistent with the accessible knowledge is assumed, and the assumption is checked through an iterative protocol. The test is based on the frequency analysis of the response of a targeted reactive species to temperature modulation. We build specific functions of the frequency that are constant for the assumed mechanism and show that the graph of these functions can be drawn from appropriate data analysis. The method is general and can be applied to any complex mechanism. It is here illustrated in detail in the case of single relaxation time mechanisms.

1. INTRODUCTION

Life sciences are presently dominated by analytic methods which consist in identifying all the relevant parts of the living machinery. Large scale approaches include genomics¹ and proteomics.² To unravel biological functions, one scrutinizes molecular interactions to establish what is called the interactome. Techniques like chromatin immunoprecipitation (ChIP)³ and yeast two-hybrid systems⁴ are used, and proximity between partners can be asserted using fluorescence resonant energy transfer measurements.^{5,6} Thus, diagrams describing the relations between numerous species can be sketched. However, the biological pathways hardly account for dynamics and evidencing some interactions is not sufficient to elucidate a chemical mechanism. The limitations of such static views appear especially in pharmacology, where the importance of binding kinetics is more and more recognized.^{7–9} Long residence times are usually associated with better efficiency, less frequent administration, and lower off-target toxicity of drugs. However, rapid dissociation can result in a better clinical

tolerability, as it has been proved for instance for nonsteroidal anti-inflammatory medicines and for molecules improving the memory-related symptoms of Alzheimer's disease.¹⁰ The number of reactants and products, as well as the associated stoichiometric coefficients, the presence of autocatalysis,^{11–14} and the value of the relaxation times^{15,16} are all relevant features to understand the role of a given chemical transformation in a biological process. Their determination would consequently improve our knowledge in fields such as drug discovery,^{8,9} protein folding,^{17,18} and systems biology.¹⁹ Direct characterization of biomolecule dynamics is therefore essential.

The sensitivity of chemical reactions to temperature has been exploited for more than 50 years in kinetic studies utilizing various T-jump apparatus^{15,20,21,18,22,23} and, more recently, techniques based on temperature oscillations.²⁴ The develop-

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ment in thermal microtechnologies²⁵ and fluorescence detection^{25,26} has significantly improved the efficiency of the temperature modulation approach. The upper accessible frequency has been shifted from initially 10^{-2} Hz²⁴ to 1 kHz,^{27,28} yielding a spectral domain overlapping the one of stopped-flow mixing.^{18,29} The use of acquisition protocols similar to the ones for lock-in amplifiers enables an easy access to the amplitude and phase of concentration oscillations with a high signal-to-noise ratio.^{30,27,31,32,28} Finally, the fact that such investigations can be performed in cellulo^{27,33} is of special interest with respect to the comprehension of living systems.

If practical issues relative to studies on biomolecule dynamics are routinely overcome, only a small number of theoretical methods for mechanism identification have been developed.^{34,35} Usually, the response to a perturbation is analyzed and dynamical parameters are deduced by fitting the experimental data in the framework of some asserted mechanism.^{16,29,36,37} However, these methods can lead to incorrect interpretation because the assumption made when the mechanism was proposed has not been rigorously checked. In addition, estimating the precision of the results is difficult when fitting parameters are used. In the case of a known mechanism of Michaelis–Menten type,³⁸ we already designed a technique to deduce the rate constants from the response to small temperature oscillations.³⁹ Here, we describe an iterative method, first, to find the mechanism obeyed by a reactive system and, second, to determine all the parameters characterizing the corresponding dynamics without resorting to fitting methods. The method is illustrated in detail for a one-relaxation time mechanism. The generalization to any complex mechanism is straightforward, even if the analytical calculations may be more tedious.

The paper is organized as follows. In section 2, we assume that the observable chemical species A reacts according to the mechanism $A + B = C$. The amplitude and phase of the concentration oscillations of species A due to a small temperature modulation are supposed to be measurable. We calculate the response of species A as a function of the excitation frequency ω and of dynamical parameters of the $A + B = C$ reaction. Reciprocally, we express some of these dynamical parameters characterizing the supposed mechanism as functions of the observable quantities. From these expressions, we build three functions of the frequency, $F(\omega)$, $G(\omega)$, and $H(\omega)$, that are designed to be constant if the checked mechanism is actually $A + B = C$. In section 3, we show that these functions are not all constant if the checked mechanism differs from $A + B = C$. In the case where the initial hypothesis is confirmed, we express the two rate constants and the two activation energies of the reaction $A + B = C$ as functions of observable quantities. In the case where the initial hypothesis is rejected, the behavior of the functions $F(\omega)$, $G(\omega)$, and $H(\omega)$ give some hints to infer a new proposition of mechanism, with which the protocol can be followed again. Section 4 is devoted to conclusion.

2. METHOD

In this section, we illustrate the method of mechanism identification in the case of a chemical reaction which is known to involve at least two species A and B. We consider a mixture where species B is in great excess compared to A, so that its concentration, B, can be considered constant. A controlled quantity of species A has been introduced in the system and the initial concentration of A before any reaction is

denoted N. We admit that the concentration of A can be measured, for example because A has specific spectroscopic properties with respect to the other compounds. For instance, A may be tagged with a fluorescent probe for which intensity, lifetime, or polarizability is modified upon reaction with B.⁵ The equilibrium concentration A^0 of species A is determined. To reveal the dynamics of the chemical system, we suggest to apply a small temporal modulation of temperature T around the value T_0 and at the angular frequency ω ,^{40,39}

$$T = T_0[1 + \beta \sin(\omega t)] \quad (1)$$

where $\beta \ll 1$, such that βT_0 is on the order of 1 deg, which prevents the denaturation of the chemical species.

The first step consists in assuming a chemical mechanism. As an example, we choose



which we simply write as $A + B = C$. The sensitivity of the system to a small excitation is ensured by the exponential dependence of the rate constants on temperature:

$$k_{\pm 1} = r_{\pm 1} \exp\left(-\frac{E_{\pm 1}}{RT}\right) \quad (3)$$

where R is the gas constant, $r_{\pm 1}$ are the pre-exponential factors in the Arrhenius equation, and $E_{\pm 1}$ are the activation energies. In the following, we use the reduced activation energies $\epsilon_{\pm 1} = E_{\pm 1}/RT_0$. Taking the experimental constraints into account and the possibility to detect signals oscillating at ω and 2ω , we consider an expansion of the rate constants to the second order in terms of the small parameter β :

$$k_{\pm 1} = k_{\pm 1}^0 + \beta k_{\pm 1}^1 + \beta^2 k_{\pm 1}^{(2)} \quad (4)$$

with $k_{\pm 1}^0 = r_{\pm 1} \exp(-\epsilon_{\pm 1})$, $k_{\pm 1}^1 = k_{\pm 1}^0 \epsilon_{\pm 1} \sin(\omega t)$, and $k_{\pm 1}^{(2)} = k_{\pm 1}^0 [\epsilon_{\pm 1}(\epsilon_{\pm 1} - 2)] \times [1 - \cos(2\omega t)]/4$, where the exponents 0, 1, (2) indicate the order of the β -expansion. The temperature modulation induces oscillations of the concentration A of species A, which can be written as

$$A = A^0 + \beta A^1 + \beta^2 A^{(2)} \quad (5)$$

with $A^1 = A^{1\sin} \sin(\omega t) + A^{1\cos} \cos(\omega t)$ and $A^{(2)} = A^{2\sin} \sin(2\omega t) + A^{2\cos} \cos(2\omega t)$. The first-order amplitudes of the concentration modulation, $A^{1\sin}$ and $A^{1\cos}$, are in phase and out of phase with the temperature, respectively. Similarly, $A^{2\sin}$ and $A^{2\cos}$ are the second-order amplitudes of the concentration modulation at the frequency 2ω . Here, we omit the constant second-order out-of-equilibrium shift, $\beta^2 A^{2\text{cst}}$, which is negligible with respect to the equilibrium value A^0 .⁴⁰

In a second step, we use the laws of kinetics to determine the expressions of the equilibrium value A^0 , the first-order in-phase and out-of-phase amplitudes, $A^{1\sin}$ and $A^{1\cos}$, the corresponding second-order amplitudes, $A^{2\sin}$ and $A^{2\cos}$, as functions of the dynamical parameters, temperature T_0 , and angular frequency ω . For the mechanism given in eq 2, we find

$$\frac{dA}{dt} = UA + V \quad (6)$$

with $U = -(k_{+1}B + k_{-1})$ and $V = k_{-1}N$, where N is the initial concentration of species A. The conservation relation $N = C + A$ has been used to eliminate the concentration C of species C in eq 6. Expanding $U = U^0 + \beta U^1 + \beta^2 U^{(2)}$ and $V = V^0 + \beta V^1 +$

$\beta^2 V^{(2)}$ to the second order in β , we solve eq 6 order by order. The parameter U of the linear term is related to the chemical relaxation time τ_1 by $\tau_1 = -1/U^0$. To the zeroth order, we straightforwardly compute

$$A^0 = \frac{k_{-1}^0 N}{k_{+1}^0 B + k_{-1}^0} \quad (7)$$

$$C^0 = N - A^0 \quad (8)$$

Then, to the first order, we obtain the equation

$$\frac{dA^1}{dt} = U^0 A^1 + U^1 A^0 + V^1 \quad (9)$$

which leads to

$$A^{1\sin} = -\frac{k_{+1}^0 B k_{-1}^0 N}{(k_{+1}^0 B + k_{-1}^0)^2 + \omega^2} \frac{\Delta_1 H}{RT_0} \quad (10)$$

$$A^{1\cos} = -\frac{\omega}{k_{+1}^0 B + k_{-1}^0} A^{1\sin} \quad (11)$$

$\Delta_1 H = E_{+1} - E_{-1}$ being the enthalpy of reaction. Finally, to the second order, eq 6 gives

$$\frac{dA^{(2)}}{dt} = U^0 A^{(2)} + U^1 A^1 + U^{(2)} A^0 + V^{(2)} \quad (12)$$

which yields

$$A^{2\sin} = \frac{(k_{+1}^0 B \epsilon_{+1} + k_{-1}^0 \epsilon_{-1})(2\omega A^{1\sin} - (k_{+1}^0 B + k_{-1}^0) A^{1\cos})}{2((2\omega)^2 + (k_{+1}^0 B + k_{-1}^0)^2)} - \frac{\omega k_{+1}^0 B A^0 (\epsilon_{-1}(\epsilon_{-1} - 2) - \epsilon_{+1}(\epsilon_{+1} - 2))}{2((2\omega)^2 + (k_{+1}^0 B + k_{-1}^0)^2)} \quad (13)$$

$$A^{2\cos} = \frac{k_{+1}^0 B + k_{-1}^0}{2\omega} A^{2\sin} + \frac{k_{+1}^0 B \epsilon_{+1} + k_{-1}^0 \epsilon_{-1}}{4\omega} A^{1\cos} \quad (14)$$

Equations 10, 11, 13, and 14 can be used to make more precise the conditions under which the chemical system significantly responds to the temperature modulation: (i) the enthalpy of reaction $\Delta_1 H$ should be sufficiently large, (ii) the reciprocal of the chemical relaxation time, $1/\tau_1 = k_{+1}^0 B + k_{-1}^0$, should belong to the range of accessible frequencies for the temperature modulation, and (iii) the concentration B should be chosen such that $k_{+1}^0 B$ is close to k_{-1}^0 , which results in an equilibrium concentration A^0 close to $N/2$.

In a third step, three independent physical quantities, a function of the reduced activation energies, $\epsilon_{-1}(\epsilon_{-1} - 2) - \epsilon_{+1}(\epsilon_{+1} - 2)$, the chemical relaxation time, τ_1 , and the enthalpy of reaction, $\Delta_1 H$, can be expressed in terms of the oscillation frequency and experimental data such as N , A^0 , and the amplitudes of oscillation of species A concentration. These expressions are used to design functions of the frequency ω that are constant by construction for the supposed mechanism but may vary for other mechanisms. Using eqs 7, 10, 11, 13, and 14, we define the three following functions:

$$F(\omega) = \frac{4N}{A^0(N - A^0)} \left(A^{2\sin} \left(\frac{2A^{1\cos}}{A^{1\sin}} - \frac{A^{1\sin}}{A^{1\cos}} \right) - 3A^{2\cos} \right) \quad (15)$$

$$G(\omega) = -\frac{A^{1\cos}}{\omega A^{1\sin}} \quad (16)$$

$$H(\omega) = -\frac{NA^{1\sin}}{A^0(N - A^0)} \left(1 + \left(\frac{A^{1\cos}}{A^{1\sin}} \right)^2 \right) \quad (17)$$

which are respectively equal to $\epsilon_{-1}(\epsilon_{-1} - 2) - \epsilon_{+1}(\epsilon_{+1} - 2)$, τ_1 , and $\Delta_1 H/RT_0$ if the probed mechanism can be identified with the target $A + B = C$.

In the next section, we draw the graphs of the three functions $F(\omega)$, $G(\omega)$, and $H(\omega)$ for various mechanisms. The analytical expressions of the first- and second-order amplitudes used to compute the values of the functions are given in Appendix A for a general mechanism with one relaxation time, and in Appendix B for a mechanism with two relaxation times.

3. RESULTS

3.1. Identification of the Mechanism. Our goal is to show that the graph of the three functions $F(\omega)$, $G(\omega)$, and $H(\omega)$ makes it possible to ascertain that the probed mechanism is of the $A + B = C$ type. The class of $A + B = C$ mechanisms regroups the isomerization reaction $A = C$ and the set of reactions deduced from it by adding reactants and products, the concentration of which is maintained constant. We use analogous notations for the other considered mechanisms. Obviously, the stoichiometry of the reagents and products in excess cannot be determined by this method.

The $A + B = C$ mechanism, with B in excess, possesses a single chemical relaxation time and a linear dynamics with respect to concentration. First, we check if the actual mechanism differs from the assumed one because it possesses more than one relaxation time. We consider the mechanism $A + B = C = D = A + B$, with B in excess, as an example of linear dynamics with two relaxation times. The association of species A and B gives the product C through the passage by an intermediate species D . This scheme, where A plays the role of the catalyst, is usual in enzymatic catalysis and known as the Michaelis–Menten mechanism.³⁸ Second, if the mechanism proves to possess a single relaxation time, we wish to differentiate it from other mechanisms with one relaxation time but nonlinear kinetics, which are commonly encountered in chemistry and biology.⁴¹ In particular, we will consider the mechanisms $2A + B = C$ and $A + B = 2C$ with different stoichiometric coefficients for species A and C , and the mechanism $A + B + C = 2C$ with an autocatalytic formation of species C . If we consider reactions between proteins, the mechanism $2A = C$ would correspond to the formation of a homodimer. Similarly, the formation of a heterodimer would be written as $A + B = C$.^{17,18,42} In living systems, autocatalysis is a generic phenomenon associated with the emergence of temporal or spatial organization.^{11–14} The autocatalytic case $A + C = 2C$ could model the propagation of a prion disease.⁴³

To determine the discriminating power of $F(\omega)$, $G(\omega)$, and $H(\omega)$ defined in eqs 15–17, we evaluate these functions for the different considered mechanisms. Instead of using experimental results for the amplitudes of oscillation of species A , we solve the differential equations governing the dynamics of each mechanism and use the analytical expressions of the amplitudes to calculate the values of the functions. The expressions of A^0 , $A^{1\sin}$, $A^{1\cos}$, $A^{2\sin}$, and $A^{2\cos}$ as functions of the rate constants, activation energies and frequency are given in Appendix A for the mechanisms $2A + B = C$, $A + B = 2C$, $A + B + C = 2C$ and in Appendix B for $A + B = C = D = A + B$.

As seen in Figure 1, the function $F(\omega)$ possesses characteristic behaviors depending on the type of mechanism. By

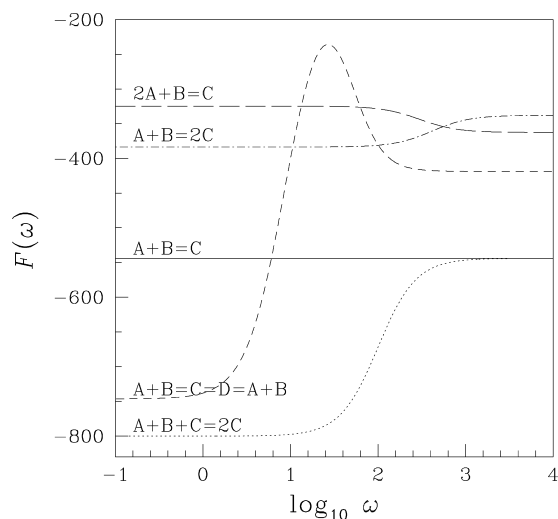


Figure 1. Graph of the function $F(\omega)$ defined in eq 15 for different reaction mechanisms. For the mechanisms with one relaxation time, the parameter values are $k_{+1}^0 B = 100$, $k_{-1}^0 = 100$, $\epsilon_{+1} = 26$, $\epsilon_{-1} = 10$, and $N = 1$. For $A + B = C = D = A + B$, we have chosen $k_{+1}^0 B = 100$, $k_{-1}^0 = 100$, $k_{+2}^0 = 10$, $k_{-2}^0 = 10$, $k_{+3}^0 = 1$, $k_{-3}^0 B = 1$, $\epsilon_{+1} = 26$, $\epsilon_{-1} = 10$, $\epsilon_{+2} = 22$, $\epsilon_{-2} = 19$, $\epsilon_{+3} = 15$, $\epsilon_{-3} = 34$, and $N = 1$. $F(\omega)$ is constant only if the probed mechanism is of $A + B = C$ type.

construction, $F(\omega)$ is constant for $A + B = C$. For the other mechanisms with a single relaxation time, $F(\omega)$ is monotonous with a single jump. The decreasing or increasing nature of $F(\omega)$ can be used to give some hints about the probed mechanism (see Appendix A).

For $A + B = C = D = A + B$, $F(\omega)$ possesses two inflection points for values of ω close to the two relaxation times. For the phenomenon to be observable, the two relaxation times must be obviously distinct, i.e., differ by an order of magnitude. Depending on the parameter values, $F(\omega)$ may be monotonous or possess an extremum. Nonetheless, two jumps associated with the two relaxation times will be observed. This result can be generalized to a mechanism with n relaxation times that differ by an order of magnitude. Then, the function $F(\omega)$ possesses n jumps which inflection points give the order of magnitude of the relaxation times.⁴⁰

The determination of the graph of the function $F(\omega)$ given in eq 15 and the observation of constant values as ω varies are therefore sufficient to ascertain that the probed mechanism is of the $A + B = C$ type. Contrary to $F(\omega)$, the functions $G(\omega)$ and $H(\omega)$ only involve the first-order amplitudes $A^{1\sin}$ and $A^{1\cos}$. As seen in Figures 2 and 3, the behavior of these functions does not help in discriminating between linear and nonlinear dynamics: Constant values, independent of ω , are obtained for all the mechanisms with a single relaxation time. A single jump is observed for the mechanism $A + B = C = D = A + B$ with two relaxation times. The behavior of $G(\omega)$ and $H(\omega)$ can be used to confirm the result deduced from $F(\omega)$, in particular, to exclude that the probed mechanism possesses more than one relaxation time.

In summary, the design of the sole function $F(\omega)$ from the amplitudes of the concentration oscillations of a single species A according to eq 15 enables us to assign, without ambiguity, the $A + B = C$ type to the probed mechanism. Experimentally, the detection of a 10% variation can be achieved.

If the procedure would lead to the rejection of the hypothesis, another chemical mechanism would be assumed

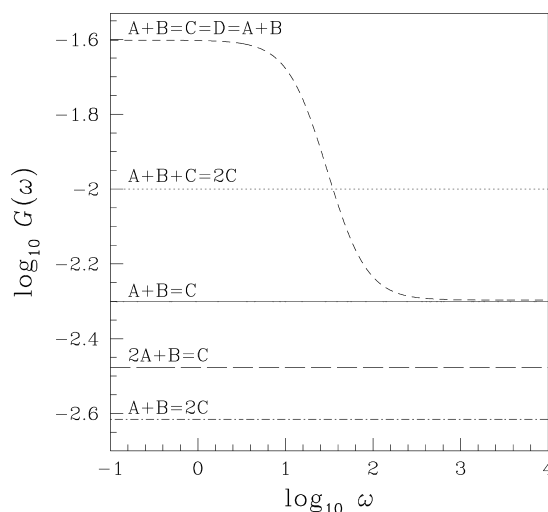


Figure 2. Graph of the function $G(\omega)$ defined in eq 16 for different reaction mechanisms and the same parameter values as in Figure 1. For the mechanisms with a single relaxation time, $G(\omega)$ gives the value of this relaxation time.

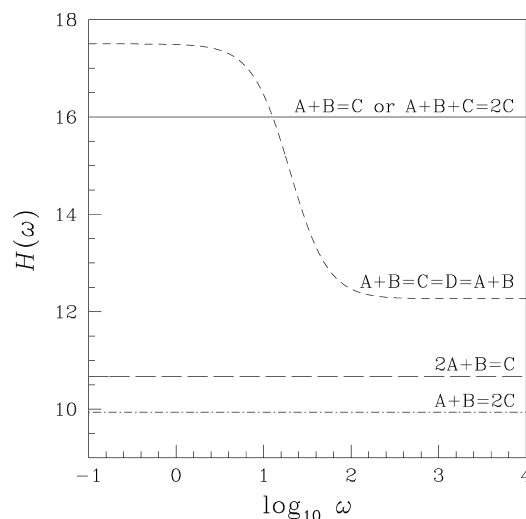


Figure 3. Graph of the function $H(\omega)$ defined in eq 17 for different reaction mechanisms and the same parameter values as in Figure 1. For $A + B = C$ and $A + B + C = 2C$, $H(\omega)$ gives the value of the enthalpy of reaction.

and the different steps would be followed again: resolution of the differential equations governing chemical kinetics, construction of a new function $F^*(\omega)$ that identifies with $\epsilon_{-1}(\epsilon_{-1} - 2) - \epsilon_{+1}(\epsilon_{+1} - 2)$ for the new assumed mechanism, measurement of N , A^0 , and the amplitudes of oscillation of species A in a wide range of frequencies for the probed chemical system, and analysis of the graph of $F^*(\omega)$.

3.2. Determination of the Thermodynamic and Kinetic Parameters. The evaluation of $F(\omega)$, $G(\omega)$, and $H(\omega)$ has revealed that the equilibrium concentration A^0 of species A and the in-phase ($A^{1\sin}$) and out-of-phase ($A^{1\cos}$) amplitudes of oscillation with temperature, to the first ($i = 1$) and second ($i = 2$) orders, can be expressed as functions of the excitation frequency ω , the reduced activation energies $\epsilon_{\pm 1}$, and the rate constants $k_{\pm 1}^0$ at temperature T_0 . Reciprocally, the determination of the response of the chemical system to temperature modulation, e.g., the measurement of N , A^0 , $A^{1\sin}$,

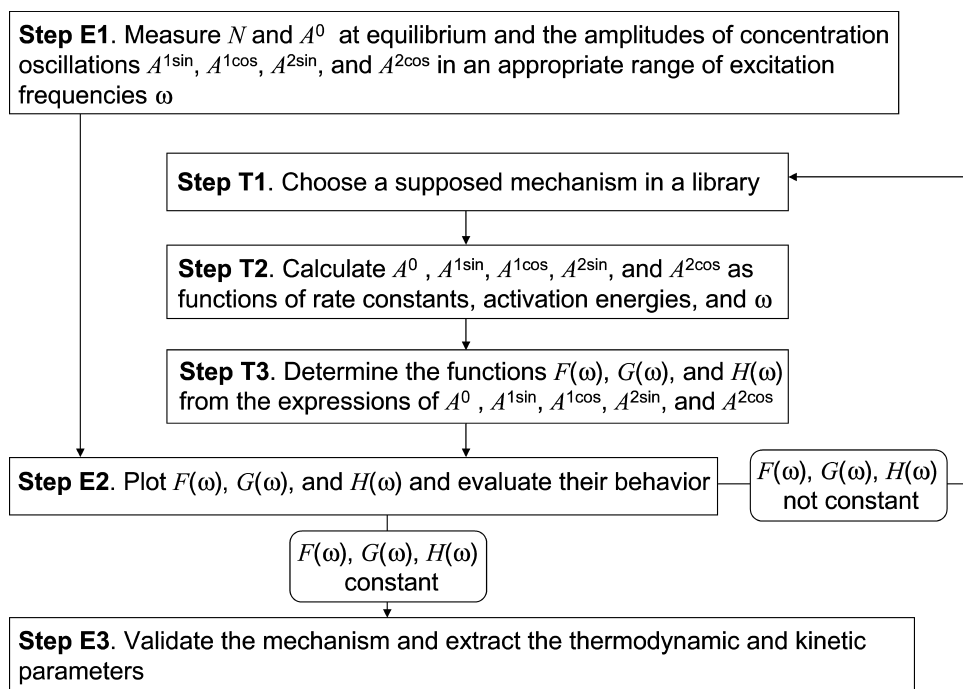


Figure 4. Protocol to be followed to determine a chemical mechanism. Steps T1, T2, and T3, where T stands for theory, consist of the determination of the functions $F(\omega)$, $G(\omega)$, and $H(\omega)$, which identify with the quantities $\epsilon_{-1}(\epsilon_{-1} - 2) - \epsilon_{+1}(\epsilon_{+1} - 2)$, τ_1 and $\Delta_1 H/RT_0$, respectively, when the actual mechanism matches the supposed one. Steps E1, E2, and E3, where E stands for experiments, consist of measurements of the observables and data processing that leads back to step T1 or moves forward to conclusive step E3.

and $A^{i\cos}$ for $i = 1, 2$, provides access to the rate constants $k_{\pm 1}^0$ and to the activation energies $\epsilon_{\pm 1}$. It is then straightforward to deduce the relaxation time τ_1 , the equilibrium constant $K_1 = k_{+1}^0/k_{-1}^0$, and the enthalpy of reaction $\Delta_1 H$.

If the probed mechanism proves to be actually $A + B = C$, eqs 7, 10, and 11 can be used to determine the dynamical parameters from experimentally accessible quantities. The two rate constants and the enthalpy of reaction are given by

$$k_{+1}^0 B = -\omega \frac{N - A^0}{N} \frac{A^{1\sin}}{A^{1\cos}} \quad (18)$$

$$k_{-1}^0 = -\omega \frac{A^0}{N} \frac{A^{1\sin}}{A^{1\cos}} \quad (19)$$

$$\frac{\Delta_1 H}{RT_0} = -\frac{NA^{1\sin}}{A^0(N - A^0)} \left(1 + \left(\frac{A^{1\cos}}{A^{1\sin}} \right)^2 \right) \quad (20)$$

Equivalently, the equilibrium constant and the relaxation time obey

$$K_1 = \frac{N - A^0}{A^0 B} \quad (21)$$

$$\tau_1 = -\frac{A^{1\cos}}{\omega A^{1\sin}} \quad (22)$$

Using the same approach as the one followed to define the function $F(\omega)$, we find

$$\begin{aligned} & \epsilon_{-1}(\epsilon_{-1} - 2) - \epsilon_{+1}(\epsilon_{+1} - 2) \\ &= \frac{4N}{A^0(N - A^0)} \left(A^{2\sin} \left(\frac{2A^{1\cos}}{A^{1\sin}} - \frac{A^{1\sin}}{A^{1\cos}} \right) - 3A^{2\cos} \right) \end{aligned} \quad (23)$$

Then, the reduced activation energies are given by

$$\epsilon_{\pm 1} = -\frac{\epsilon_{-1}(\epsilon_{-1} - 2) - \epsilon_{+1}(\epsilon_{+1} - 2)}{\frac{2\Delta_1 H}{RT_0}} \pm \frac{\Delta_1 H}{2RT_0} + 1 \quad (24)$$

where $\Delta_1 H/RT_0$ and $\epsilon_{-1}(\epsilon_{-1} - 2) - \epsilon_{+1}(\epsilon_{+1} - 2)$ are given by eqs 20 and 23, respectively.

3.3. Experimental Protocol. The different steps of the protocol to be followed to assign a given mechanism to a chemical system are presented in Figure 4. The procedure begins with the experimental step E1 and the measurements of the different observables. After the global fluorescent signal are collected, the different in-phase and out-of-phase amplitudes at the first and second orders in the perturbation, $A^{1\sin}(\omega)$, $A^{1\cos}(\omega)$, $A^{2\sin}(\omega)$, and $A^{2\cos}(\omega)$, must be extracted. Lock-in amplifiers, also called phase-sensitive detectors, are used to determine the amplitudes and phase shift with respect to temperature modulation with a high signal-to-noise ratio. In parallel, the theoretical steps T1, T2, and T3 must be followed to assume a mechanism, calculate the expressions of the observables as functions of the frequency ω and the thermodynamic and kinetic parameters, and find the functions $F(\omega)$, $G(\omega)$, and $H(\omega)$ in terms of the observables. Then, in step E2, the graph of these three functions is plotted using the theoretical expressions obtained in step T3 and the data collected in step E1. Depending on the behavior of $F(\omega)$, $G(\omega)$, and $H(\omega)$, the supposed mechanism is either rejected or validated. In the first case, the theoretical procedure has to be followed again from step T1. In the second case, the thermodynamic and kinetic parameters are evaluated in step E3.

Table 1. Equilibrium Concentration of Species A for the Different Mechanisms Considered in the Figures

	2A + B = C	A + B = 2C	A + B + C = 2C
A^0	$\frac{-k_{-1}^0 + \sqrt{(k_{-1}^0)^2 + 8k_{+1}^0 B k_{-1}^0 N}}{4k_{+1}^0 B}$	$\frac{8k_{-1}^0 N + k_{+1}^0 B(1 - \sqrt{1 + 4k_{-1}^0/k_{+1}^0 B})}{8k_{-1}^0}$	$\frac{Nk_{-1}^0}{k_{+1}^0 B + k_{-1}^0}$

4. CONCLUSION

In this paper, we present a method to fully characterize a reaction whose mechanism is initially unknown. Compatible with available technologies, the protocol requires that at least one species involved in the probed mechanism is detectable, for example, due to its fluorescence properties. Small temperature oscillations are imposed and the frequency response of the detectable species is analyzed in a wide range of oscillation frequency. The oscillations of concentration to the first and second orders with respect to the perturbation contain enough information to check if the probed system reacts with a given supposed mechanism. The method relies on the design of functions of the frequency that are constant if the probed mechanism identifies with the supposed one. In the case of success, the same observable quantities are used to determine the kinetic and thermodynamic parameters associated with the confirmed mechanism. In the case of failure, the analysis of the graph of the functions gives some hints to infer a new supposed mechanism, so that the entire protocol can be followed again. These results evidence the richness of the response of a reactive system to a small temperature modulation. This noninvasive approach is well adapted to answer a specific question of biological relevance.

■ APPENDIX A: REACTION WITH A SINGLE RELAXATION TIME

In this section we derive the expressions of the equilibrium concentration, the in-phase and out-of-phase amplitudes to the first and second orders of a detectable species involved in a generic reaction with one relaxation time. These expressions have been used to draw the graph of the functions $F(\omega)$, $G(\omega)$, and $H(\omega)$ in Figures 1–3 for some probed mechanisms.

A.1. First- and Second-Order Responses of Species A

Any mechanism describing the reaction between species A and B forming at least one product, C, and possibly another one, D, can be written in the general form



A specific mechanism is obtained by assigning the appropriate set of values for the stoichiometric coefficients (a , γ , c , d). For example, the mechanism $2A + B = C$ is obtained when the set ($a = 2$, $\gamma = 0$, $c = 1$, $d = 0$) is assigned to eq 25. Species B being in excess, its concentration is constant. Consequently, the stoichiometric coefficient b of species B does not influence the dynamics and we impose the value $b = 1$. The laws of chemical kinetics lead to

$$\frac{1}{a} \frac{dA}{dt} = -k_{+1} B A^a C^\gamma + k_{-1} C^c D^d \quad (26)$$

Initially, species A is introduced in the system at concentration N . At equilibrium, the concentration of species A reaches the value A^0 . Using the conservation of matter, we find $C^0 = [(c - \gamma)/a](N - A^0)$ and $D^0 = (d/a)(N - A^0)$. The law of mass action can be written as

$$\frac{k_{+1}^0 B}{k_{-1}^0} = \frac{d^d (c - \gamma)^{c-\gamma} (N - A^0)^{d+c-\gamma}}{a^{d+c-\gamma} (A^0)^a} \quad (27)$$

Instead of the cumbersome expression of the equilibrium concentration for the general mechanism of eq 25, Table 1 gives the results for the particular cases considered in the figures. Once the equilibrium is reached, the experimental system containing the different species A, B, C, and D is submitted to a small temperature modulation, the expression of which is given in eq 1. The oscillating response concentration of species A can be decomposed as given in eq 5. Solving eq 26 at the first and second orders in β , we determine the expressions of the first- and second-order response amplitudes

$$A^{1\sin} = \frac{\tau_1}{1 + (\omega\tau_1)^2} \nu_0 (\varepsilon_{-1} - \varepsilon_{+1}) \quad A^{1\cos} = -\omega\tau_1 A^{1\sin} \quad (28)$$

$$A^{2\sin} = \frac{2\omega\tau_1}{1 + 4(\omega\tau_1)^2} \left(\frac{3\nu_1}{4} A^{1\sin} + \frac{\nu_{11}}{2} (2 - (\omega\tau_1)^2) (A^{1\sin})^2 - \nu_2 \right) \quad (29)$$

$$A^{2\cos} = \frac{1}{2\omega\tau_1} A^{2\sin} + \frac{\nu_1}{4\omega} A^{1\cos} + \frac{\nu_{11}}{2\omega} A^{1\cos} A^{1\sin} \quad (30)$$

where $\nu_0 = ak_{+1}^0 B (A^0)^a (C^0)^\gamma$. The expressions of the different parameters τ_1 , ν_1 , ν_{11} , and ν_2 as functions of the stoichiometric coefficients, equilibrium concentrations, rate constants, and activation energies are

$$\tau_1 = \frac{1}{\nu_0 \left(\frac{a}{A^0} + \frac{(c-\gamma)^2}{aC^0} + \frac{d^2}{aD^0} \right)} \quad (31)$$

$$\nu_1 = \nu_0 \left(\varepsilon_{+1} \left(\frac{a}{A^0} - \frac{\gamma}{N - A^0} \right) + \varepsilon_{-1} \frac{d + c}{N - A^0} \right) \quad (32)$$

$$\nu_{11} = \frac{\nu_0}{2} \left(\frac{a(a-1)}{(A^0)^2} - \frac{2\gamma a}{A^0(N - A^0)} + \frac{\gamma(\gamma-1) - 2cd - c(c-1) - d(d-1)}{(N - A^0)^2} \right) \quad (33)$$

$$\nu_2 = \frac{\nu_0}{4} (-\varepsilon_{+1}(\varepsilon_{+1} - 2) + \varepsilon_{-1}(\varepsilon_{-1} - 2)) \quad (34)$$

A.2. Expression of the Test Functions

Using the expressions of the first- and second-order amplitudes of species A concentration given in eqs 28–30, we find the general expression of the functions $F(\omega)$, $G(\omega)$, and $H(\omega)$ defined in eqs 15–17 for a species A involved in a one-relaxation time reaction. The functions $G(\omega)$ and $H(\omega)$ are respectively equal to

$$G(\omega) = \tau_1 \quad (35)$$

$$H(\omega) = \frac{N\nu_0\tau_1}{A^0(N-A^0)} \frac{\Delta_1 H}{RT_0} \quad (36)$$

where τ_1 is given in eq 31. Both are independent of ω and the function G is equal to the relaxation time of the reaction, regardless of the mechanism. The value of H is proportional to the enthalpy of reaction.

Substituting $A^{2\sin}$, $A^{2\cos}$, $A^{1\sin}$, and $A^{1\cos}$ with their expressions given in eqs 28–30 into the expression of $F(\omega)$ given in eq 15, we find

$$F(\omega) = \frac{4N\tau_1}{A^0(N-A^0)} \left(v_2 + \frac{\nu_{11}\tau_1^2\nu_0^2}{2(1+(\omega\tau_1)^2)} \left(\frac{\Delta_1 H}{RT_0} \right)^2 \right) \quad (37)$$

where ν_{11} and ν_2 are given in eqs 33 and 34. For the mechanism $A + B = C$ with B in excess ($a = 1$, $c = 1$, $\gamma = 0$, $d = 0$), the coefficient ν_{11} vanishes. In this case, the function $F(\omega)$ is constant. For all the other mechanisms with one relaxation time, the reaction rate is a nonlinear function of the concentrations. Consequently, the parameter ν_{11} does not vanish and $F(\omega)$ presents a threshold for $\omega = 1/(3^{1/2}\tau_1)$. The height of the threshold between high and low frequencies, $F(\infty) - F(0)$, is equal to

$$F(\infty) - F(0) = -\frac{2N\nu_{11}\tau_1^3}{A^0(N-A^0)} \nu_0^2 \left(\frac{\Delta_1 H}{RT_0} \right)^2 \quad (38)$$

The sign of $F(\infty) - F(0)$ is imposed by the one of $-\nu_{11}$. For the mechanisms $A + B = 2C$ and $A + B + C = 2C$, the coefficient ν_{11} is negative and the height of the threshold $F(\infty) - F(0)$ is positive, whereas for $2A + B = C$, ν_{11} is positive and $F(\infty) - F(0)$ is negative, as represented in Figure 1. The observation of a threshold in the graph of $F(\omega)$ confirms that the probed mechanism is not the expected one, i.e., that the hypothesis $A + B = C$ has to be rejected. Another proposition of mechanism has to be formulated. For example, if the graph of $F(\omega)$ has revealed a decreasing function, the next step will consist in checking if the mechanism is $2A + B = C$. To this goal, three new functions F^* , G^* , and H^* must be defined from the expressions of $\epsilon_{-1}(\epsilon_{-1} - 2) - \epsilon_{+1}(\epsilon_{+1} - 2)$, τ_1 , and $\Delta_1 H/RT_0$ for the mechanism $2A + B = C$, so that they are constant by construction if the probed mechanism is actually $2A + B = C$.

Once a mechanism has been confirmed, i.e., for known values of the stoichiometric coefficients (a , c , γ , d), the different parameters governing the dynamics can be determined from experimental data. We find

$$k_{+1}^0 B = -\omega \frac{A^{1\sin}}{A^{1\cos}} \frac{1}{aw_0(A^0)^a \left(\frac{c-\gamma}{a}(N-A^0) \right)^\gamma} \quad (39)$$

$$k_{-1}^0 = -\omega \frac{A^{1\sin}}{A^{1\cos}} \frac{1}{aw_0 \left(\frac{d}{a}(N-A^0) \right)^d \left(\frac{c-\gamma}{a}(N-A^0) \right)^c} \quad (40)$$

$$\frac{\Delta_1 H}{RT_0} = w_0 A^{1\sin} \left(1 + \left(\frac{A^{1\cos}}{A^{1\sin}} \right)^2 \right) \quad (41)$$

$$\begin{aligned} & \epsilon_{-1}(\epsilon_{-1} - 2) - \epsilon_{+1}(\epsilon_{+1} - 2) \\ &= 4w_0 \left(A^{2\sin} \left(\frac{2A^{1\cos}}{A^{1\sin}} - \frac{A^{1\sin}}{A^{1\cos}} \right) - 3A^{2\cos} \right. \\ & \quad \left. - \frac{\nu_{11}}{2\nu_0 w_0} ((A^{1\sin})^2 + (A^{1\cos})^2) \right) \end{aligned} \quad (42)$$

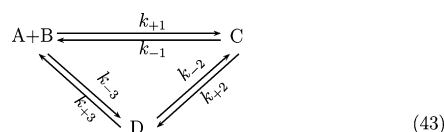
where

$$w_0 = \left(\frac{a}{A^0} + \frac{c-\gamma}{N-A^0} + \frac{d}{N-A^0} \right)$$

The two activation energies can be deduced from eq 24 and eqs 41 and 42.

■ APPENDIX B: MECHANISM WITH TWO RELAXATION TIMES

We choose to illustrate the case of a mechanism with two relaxation times by the Michaelis–Menten mechanism for enzymatic catalysis.^{38,39} This well-known mechanism can be written as $A + B = C = D = A + B$ or



Using the relation of conservation of matter, $A + C + D = N$, we find that the dynamics of the reaction network is governed by a linear system of coupled equations, which may be written in a matrix format as

$$\frac{d\mathbf{A}}{dt} = \mathbf{U}\mathbf{A} + \mathbf{V} \quad (44)$$

with

$$\mathbf{U} = \begin{pmatrix} -(k_{+2} + k_{-1} + k_{+1}B) & k_{-2} - k_{+1}B \\ k_{+2} - k_{-3}B & -(k_{-2} + k_{+3} + k_{-3}B) \end{pmatrix}$$

$$\mathbf{A} = \begin{pmatrix} A \\ C \end{pmatrix}$$

and

$$\mathbf{V} = \begin{pmatrix} k_{+3} \\ k_{-2} \end{pmatrix}$$

The equilibrium concentration of species A is given by

$$A^0 = \frac{N}{1 + \frac{k_{+1}^0 B}{k_{-1}^0} + \frac{k_{-3}^0 B}{k_{+3}^0}} \quad (45)$$

To solve eq 44 to the first and second orders, we introduce the new coordinates:

$$\mathbf{W} = \begin{pmatrix} W_+ \\ W_- \end{pmatrix}$$

such that

$$\mathbf{A} = \mathbf{P}\mathbf{W} \quad (46)$$

the change of basis matrix \mathbf{P} can be written as

$$\mathbf{P} = \begin{pmatrix} \cos(\theta_+) & \cos(\theta_-) \\ \sin(\theta_+) & \sin(\theta_-) \end{pmatrix} \quad (47)$$

where the “eigenangles” θ_{\pm} characterize the eigendirections respectively associated with the eigenvalues λ_{\pm} of the matrix \mathbf{U} to the zeroth order.

To the first order, we find $W_{\pm}^1 = W_{\pm}^{1\sin} \sin(\omega t) + W_{\pm}^{1\cos} \cos(\omega t)$ with

$$W_{\pm}^{1\sin} = -\frac{\lambda_{\pm} \gamma_{\pm}^1}{\omega^2 + \lambda_{\pm}^2} \quad (48)$$

$$W_{\pm}^{1\cos} = -\frac{\omega \gamma_{\pm}^1}{\omega^2 + \lambda_{\pm}^2} \quad (49)$$

where

$$\begin{pmatrix} \gamma_+^1 \\ \gamma_-^1 \end{pmatrix} = \mathbf{P}^{-1} \mathbf{U}^1 \mathbf{A}^0 + \mathbf{V}^1$$

\mathbf{P}^{-1} being the inverse matrix of the change of basis matrix \mathbf{P} .

To the second order, it reads $W_{\pm}^{(2)} = W_{\pm}^{2\cos} \cos(2\omega t) + W_{\pm}^{2\sin} \sin(2\omega t)$ with

$$W_{\pm}^{2\sin} = -\frac{2\omega(\gamma_{\pm}^{\sin} + \gamma_{\pm}^{(2)}) + \lambda_{\pm} \gamma_{\pm}^{\cos}}{2((2\omega)^2 + \lambda_{\pm}^2)} \quad (50)$$

$$W_{\pm}^{2\cos} = \frac{\lambda_{\pm}(\gamma_{\pm}^{\sin} + \gamma_{\pm}^{(2)}) - 2\omega \gamma_{\pm}^{\cos}}{2((2\omega)^2 + \lambda_{\pm}^2)} \quad (51)$$

where

$$\begin{pmatrix} \gamma_+^{(2)} \\ \gamma_-^{(2)} \end{pmatrix} = \mathbf{P}^{-1} \mathbf{U}^{(2)} \mathbf{A}^0 + \mathbf{V}^{(2)}$$

and

$$\begin{pmatrix} \gamma_+ \\ \gamma_- \end{pmatrix} = \mathbf{P}^{-1} \mathbf{U}^1 \mathbf{P} \begin{pmatrix} W_+^1 \\ W_-^1 \end{pmatrix}$$

Once \mathbf{W} has been determined, it is easy to be traced back to \mathbf{A} , using eq 46.

However, in the case of a mechanism with two relaxation times such as $\mathbf{A} + \mathbf{B} = \mathbf{C} = \mathbf{D} = \mathbf{A} + \mathbf{B}$, the detection of the sole species \mathbf{A} is insufficient in view of the rate constant determination. The detection of species \mathbf{C} or \mathbf{D} and the measurement of the amplitude of its concentration oscillations is necessary.³⁹

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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