Identification of two-step chemical mechanisms using small temperature oscillations and a single tagged species

F. Closa,1,2 C. Gosse,3 L. Jullien,4,5,6 and A. Lemarchand1,2,a)  
1Sorbonne Universités, UPMC Univ. Paris 06, Laboratoire de Physique Théorique de la Matière Condensée,  
4 place Jussieu, case courrier 121, 75252 Paris Cedex 05, France  
2CNRS, UMR 7600 LPTMC, 75005 Paris, France  
3Laboratoire de Photonique et de Nanostructures, LPN-CNRS, route de Nozay, 91460 Marcoussis, France  
4Department of Chemistry, Ecole Normale Supérieure - PSL Research University, 24 rue Lhomond,  
75005 Paris, France  
5Sorbonne Universités, UPMC Univ. Paris 06, PASTEUR, 75005 Paris, France  
6CNRS, UMR 8640 PASTEUR, 75005 Paris, France  

(Received 10 December 2014; accepted 20 April 2015; published online 7 May 2015)

In order to identify two-step chemical mechanisms, we propose a method based on a small temperature modulation and on the analysis of the concentration oscillations of a single tagged species involved in the first step. The thermokinetic parameters of the first reaction step are first determined. Then, we build test functions that are constant only if the chemical system actually possesses some assumed two-step mechanism. Next, if the test functions plotted using experimental data are actually even, the mechanism is attributed and the obtained constant values provide the rate constants and enthalpy of reaction of the second step. The advantage of the protocol is to use the first step as a probe reaction to reveal the dynamics of the second step, which can hence be relieved of any tagging. The protocol is anticipated to apply to many mechanisms of biological relevance. As far as ligand binding is considered, our approach can address receptor conformational changes or dimerization as well as competition with or modulation by a second partner. The method can also be used to screen libraries of untagged compounds, relying on a tracer whose concentration can be spectroscopically monitored © 2015 AIP Publishing LLC. [http://dx.doi.org/10.1063/1.4919632]

I. INTRODUCTION

The importance of dynamics in tackling biological issues is widely recognized and the challenges posed by kinetics characterization are many. Scientific fields as diverse as systems chemistry,1–3 drug discovery,4–7 ligand binding,8–11 enzymology,12,13 and protein folding14,15 are concerned. The problem can in fact be addressed at different scales, from densely connected networks associated with complex metabolic or signaling pathways1–3 to single-molecule behavior and first-passage time distributions.16 To work with chemical systems in microreactors or in cellulo, we choose an intermediate description level where the laws of chemical kinetics derived for elementary processes are appropriate to follow the evolution of the concentrations in reactive species.

At the selected scale, the experimental techniques utilized to investigate dynamics usually belong to the group of relaxation methods.17,18 They most often consist in analyzing the temporal response of the chemical system to an initial, sudden, perturbation.14,17–19 The modified control parameter can, for instance, be a concentration as in the case of stopped-flow devices13,14,20–22 and surface plasmon resonance (SPR) apparatus,23,24 or the temperature as in the case of T-jump setups.14,15,28–31 In general, these techniques provide rate constant values by fitting the acquired data points with equations derived for a given, hypothetical, mechanism. However, it has been recognized that the goodness of fit can be misleading.10,11,32 Some reactions are difficult to investigate and more sophisticated criteria may become necessary to validate a mechanism.33–35 We recently devised a novel approach able to ascertain a mechanism and to determine the associated thermokinetic parameters.36,37 As experimentally demonstrated by several research teams,38–41 dynamics can be revealed by applying small temperature modulations in a wide range of frequencies and by measuring the induced in-phase and out-of-phase amplitudes of concentration oscillations. In order to check a given mechanism, we have proposed to build test functions which take constant values if the probed chemical system actually follows the supposed mechanism. In the situation where the hypothesis is validated, the values of the test functions additionally provide the rate constants and enthalpies of reaction. We first applied these ideas to one-step mechanisms and theoretically showed that elucidating the nature of the reaction involves the detection of both first- and second-order amplitudes of oscillations for one species, i.e., of concentration modulations at the frequency and at twice the frequency of the thermal harmonic forcing.36 We also conceived a protocol for two-step mechanisms, which requires the determination of only first-order amplitudes but for two chemical species.37

In this paper, we investigate the possibility to apply the preceding methodology to two-step mechanisms for which information on the first step is accessible. More precisely, we
assumed that the two rate constants and the enthalpy of reaction of the first step have been determined using, for example, the protocol we have designed for one-step mechanisms or some of the above mentioned concentration-jump techniques (e.g., stopped-flow and SPR biosensors). Our goal is to show that, in this case, the detection of the first-order oscillation amplitudes of a single species involved in the first step is sufficient to check and entirely characterize the two-step mechanism, provided that at least one reagent is involved in both steps. Interestingly, the latter species is not necessarily the one that has to be observed.

The species whose concentration oscillations are monitored will be called the tagged species in the following. As far as real systems are considered it will often correspond to a molecule whose spectroscopic brightness is significantly modified through the transformation associated with the first chemical step. This effect can be due to a label appropriately introduced by the experimentalist (e.g., by fusion with a fluorescent protein and by conjugation with an organic dye) or it can be an intrinsic property of the reagent under study (e.g., the fluorescence emission of a tryptophan residue). Of particular significance is the case where the tagged species only participate in the first step. Indeed, the latter reaction can consequently be seen as a probe to study the tagged species without loss of generality.

Two different types of two-step mechanisms are commonly encountered in chemistry and biology. The first one corresponds to a competition between species and for reagent A. In this case, the second step is given by

$$a_2 A + b_2 B_2 \Rightarrow c_2 C_2.$$  \hspace{1cm} (2)

The second type of mechanism is a sequence of reactions where the intermediate species produced by the first step is consumed in the second one. In this case, the second step obeys

$$a_2' C_1 + b_2' B_2 \Rightarrow c_2' C_2.$$  \hspace{1cm} (3)

When such a two-step mechanism is entirely unknown, we recently showed that it was necessary to detect the oscillations of two species, for example, and for reagent B, to identify the whole mechanism: as well as needed to be tagged. In fact, the protocol described in the present article becomes relevant if a first experiment can be done in the absence of B. Hence, the first step given in Eq. (1) can be fully characterized as discussed above. Subsequently, in a second experiment, full information on the effect of B can be retrieved through the sole detection of and the knowledge acquired on Eq. (1).

Table I gives various examples of two-step mechanisms found in biology and illustrating the two presented schemes. The sextuplet of stoichiometric coefficients corresponds to a mechanism given in Eqs. (1) and (2) while the sextuplet describes a mechanism characterized by Eqs. (1) and (3).

Ligand binding often departs from a simple one-step mechanism, thereby displaying more than a single relaxation time. Limiting ourselves to two-steps reactions, the induced fit and the conformational selection mechanisms have to be considered. \hspace{1cm} (4) In the case of induced fit, the complex is first formed from and through Eq. (1) and it then undergoes an isomerization to yield according to Eq. (3). Hence, induced fit corresponds to the sextuplet . In the case of conformational selection, the protein first undergoes an isomerization to result in which next binds to ligand in agreement with the mechanism given in Eqs. (1) and (3) and with the sextuplet . In these two cases, the tagged species is the protein. However, one can imagine to observe the ligand. The protein will thus be species A; the induced fit mechanism will still be associated with whereas the conformational selection one will now correspond to . Hence, the reference to a given set of stoichiometric coefficients depends on the experimental constraints, i.e., on the choice of the most easily tagged molecule and the step for which the thermokinetic parameters can most readily be determined.

Instead of looking for ligands that bind to a single receptor, one may be interested in targeting protein-protein interactions...
TABLE I. Examples of biological systems illustrating the different mechanisms described by Eqs. (1) and (2) or Eqs. (1) and (3) on which kinetic investigations have been performed. The following notations are used: dNTP = deoxynucleoside triphosphate; mant = 2′(3′)-O-(N-methylanthranilyl); actomyosin S1 = actomyosin subfragment 1; Rep helicase = replication helicase; PABA = p-aminobenzamidine; \(^3\)RNA\(^{Phe}\) = phenylalanine transfer RNA; KIX = the protein interaction domain of the CREB-binding protein; FITC-MLL = a fluorescent isothiocyanate of the lysine (K)-specific methyltransferase 2A; pKID = the phosphorylated kinase-inducible-domain of the CREB protein; Bo-Pz = a bodipy-pirenepine conjugate; EGFp-hm1 = the enhanced green fluorescent protein fused with the human M1 muscarinic receptor; compound A = N-thiazol-2-yl-2-amino-4-fluoro-5-(1-methylimidazo1-2-yl)thiobenzamide.

<table>
<thead>
<tr>
<th>Stoichiometric coefficients</th>
<th>Mechanism</th>
<th>(B_1^i)</th>
<th>A</th>
<th>B2</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>((1, 1, 1, 1^<em>, 0^</em>, 1^*))</td>
<td>Induced fit</td>
<td>Antithrombin III</td>
<td>Heparin</td>
<td>...</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Polypeptide deformylase</td>
<td>Actinoin</td>
<td>...</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T7 DNA polymerase - DNA</td>
<td>dNTP</td>
<td>...</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mant-ATP</td>
<td>Actomyosin S1</td>
<td>...</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mant-ATP</td>
<td>Rep helicase</td>
<td>...</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>((0, 1, 1^<em>, 1^</em>, 1^*))</td>
<td>Conformational selection</td>
<td>Thrombin</td>
<td>...</td>
<td>PABA</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Glucokinase</td>
<td>...</td>
<td>Glucose</td>
<td>53 and 54</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>56</td>
</tr>
<tr>
<td>((1, 1, 1, 1, 0, 1))</td>
<td>Conformational selection</td>
<td>Mant-ADP</td>
<td>DnaC protein</td>
<td>...</td>
<td>8</td>
</tr>
<tr>
<td>((1, 1, 1^<em>, 1^</em>, 1^*))</td>
<td>Allosteric modulation</td>
<td>KIX</td>
<td>FITC-MLL</td>
<td>pKID</td>
<td>58</td>
</tr>
<tr>
<td>((1, 1, 1, 1, 1, 1))</td>
<td>Tracer binding</td>
<td>Bo-Pz</td>
<td>EGFp-hm1</td>
<td>Atropine</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Compound A</td>
<td>Glucokinase</td>
<td>LY2121260</td>
<td></td>
<td>54</td>
</tr>
</tbody>
</table>

interactions. In this situation, it would be valuable to make the difference between the case where the drug B2 prevents the formation of the C1 heteroprotein complex by fixation on partner A and the case where the pharmaceutical effect is obtained through some interaction with C1 and the production of a ternary structure. Thus, the two sets of stoichiometric coefficients one has to distinguish are here \((1, 1, 1, 1, 1)\) and \((1, 1, 1^*, 1^*, 1^*)\). The same mechanistic question arises when trying to understand if a small molecule B1 can inhibit an enzyme A by competing with its substrate \(B_1^i\) or by interacting with a site different from the binding pocket.\(^{7, 12}\)

It is worth noting that beyond the drug discovery context, the sextuplet \((1, 1, 1^*, 1^*, 1^*)\) illustrates a generic mechanism related to allostery, where binding of a first ligand to the receptor modulates the ability for a second one to associat.\(^{38, 58}\)

Finally, as already mentioned, the \((1, 1, 1, 1, 1)\) stoichiometric coefficients may correspond to a protocol where the known 1:1 complexation of a tracer molecule with a receptor is used to unravel the association mechanism involving a spectroscopically invisible ligand.\(^{42, 54}\)

III. TEST CONSTRUCTION

Following a procedure that we recently developed,\(^{36, 37}\) in line with the existing experimental corpus,\(^{38–41, 59–62}\) dynamics is revealed by analyzing the response of the chemical system to a small temporal modulation of the temperature, 

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

(4)

around the value \(T_0\) and at the angular frequency \(\omega\). Values of \(\beta\) such that \(\beta T_0\) is in the order of a few degrees are typical.\(^{36–41}\) Temperature oscillations induce oscillations of the rate constants. Assuming that the Arrhenius law is obeyed, the latter parameters are given by

\[
k_{\pm i} = r_{\pm i} \exp\left(-\frac{E_{\pm i}}{RT}\right),
\]

(5)

where \(R\) is the gas constant, \(r_{\pm i}\) are pre-exponential factors, and \(E_{\pm i}\) are activation energies \((i = 1, 2)\). To the first-order expansion in \(\beta\), we thus have

\[
k_{\pm i} = k_{\pm i}^0 + \beta k_{\pm i}^1,
\]

(6)

with \(k_{\pm i}^0 = r_{\pm i} \exp(-\varepsilon_{\pm i})\) and \(k_{\pm i}^1 = k_{\pm i}^0 \epsilon_{\pm i} \sin(\omega t)\). The \(\epsilon_{\pm i} = E_{\pm i}/(RT_0)\) are the reduced activation energies, related to the enthalpy of reaction through \(\epsilon_{\pm i} = E_{\pm i}/(RT_0)\), and the exponents 0 and 1 indicate the order of the \(\beta\)-expansion. Beyond the Arrhenius law, the present perturbative method can be extended to any temperature dependence of the \(k_{\pm i}\).\(^{63}\) Next, according to the laws of chemical kinetics, oscillations of rate constants imply oscillations of concentrations. For the tagged species and to the first order, we obtain

\[
B_1^i = B_1^i + \beta B_1^i,
\]

(7)

with

\[
B_1^i = B_1^i \sin(\omega t) + B_1^i \cos(\omega t).
\]

(8)

As mentioned in the Introduction, the method first consists in assuming a mechanism, i.e., Eqs. (1) and (2) and values of the stoichiometric coefficients \((a_1, b_1, c_1, a_2, b_2, c_2)\) or Eqs. (1) and (3) and values of \((a_1, b_1, c_1, a_1', b_1', c_1')\). Subsequently, we elaborate two test functions,\(G_2(\omega)\) and \(H_2(\omega)\), that depend on the three observables, \(B_1^i, B_1^i_{\sin}\), and \(B_1^i_{\cos}\), and are constant over a wide range of excitation frequencies if the assumed mechanism is obeyed. More precisely, if the values of \(B_1^i, B_1^i_{\sin}\), and \(B_1^i_{\cos}\) are associated with the assumed mechanism, then the two kinetic invariants \(G_2(\omega)\) and \(H_2(\omega)\) are equal to \(k_{\pm 2}\) and \(H_{\pm 2}/(RT_0)\), respectively. Incidentally, the test functions also depend on the known parameters \(k_{\pm 1}\) and \(H_{\pm 1}/(RT_0)\), the assumed stoichiometric coefficients \((a_1, b_1, c_1, a_2, b_2, c_2)\), or \((a_1', b_1', c_1')\), and the conserved masses.

As an illustration, we provide below the process leading to the analytical expression of the test functions when it is supposed that the mechanism complies with Eqs. (1) and (2) and the \((1, 1, 1, 1, 1, 1)\) sextuplet. These cumbersome computations were achieved with the help of the formal solver of Mathematica. The program which extends these results to all the
possible sextuplets \((a_1, b_1, c_1, a_2, b_2, c_2)\) and \((a_1, b_1, c_1, a'_2, b'_2, c'_2)\) is provided as supplemental material.\(^{49}\)

Chemical dynamics is governed by the following rate equations:

\[
\frac{dA}{dt} = -k_{+1}AB_1 + k_{-1}C_1 - k_{+2}AB_2 + k_{-2}C_2, \tag{9}
\]

\[
\frac{dB_1}{dt} = -k_{+1}AB_1 + k_{-1}C_1. \tag{10}
\]

Three quantities denoted by \(N, N_1,\) and \(N_2\) are conserved: mass conservation of \(A\) leads to \(N = A + (a_1/c_1)\ C_1 + (a_2/c_2)\ C_2\), mass conservation of \(B_1\) to \(N_1 = B_1 + (b_1/c_1)\ C_1\), and mass conservation of \(B_2\) to \(N_2 = B_2 + (b_2/c_2)\ C_2\). Note that the steady state of the system obeys detailed balance and is therefore an equilibrium state; however, the generalization of the method to a nonequilibrium steady state is straightforward.\(^{64}\) The zero-order equations provide the expressions of the thermodynamic constants versus the equilibrium concentrations,

\[
\frac{k^0_{+1}}{k^0_{-1}} = \frac{N_1 - B^0_1}{A^0B^0_1}, \tag{11}
\]

\[
\frac{k^0_{+2}}{k^0_{-2}} = \frac{N - N_1 + B^0_1 - A^0}{A^0(A^0 - B^0_1 + N_1 + N_2 - N)}. \tag{12}
\]

As for \(B^1_1\), the first-order concentrations of species \(A\) can be decomposed into sine and cosine terms, \(A^1 = A^{1\sin} \sin(\omega t) + A^{1\cos} \cos(\omega t)\). The first-order dynamics can therefore be written in a matrix format,

\[
\frac{d}{dt} \mathbf{P} = \mathbf{M} \mathbf{P} + \mathbf{Q} \sin(\omega t), \tag{13}
\]

where

\[
\mathbf{P} = \begin{pmatrix} A^1_1 \\ B^1_1 \end{pmatrix},
\]

\[
\mathbf{M} = \begin{pmatrix} -k^{0}_{+1}B^0_1 - k^{0}_{+2}B^0_2 - k^{0}_{+2}A^0 - k^{0}_{-2} & -k^{0}_{+1}A^0 - k^{0}_{-1} + k^{0}_{+2}A^0 + k^{0}_{-2} \\ -k^{0}_{+1}B^0_1 & -k^{0}_{+1}A^0 - k^{0}_{-1} \end{pmatrix},
\]

\[
\mathbf{Q} = \begin{pmatrix} -k^{0}_{+1}B^0_1 \Delta H^1_1/RT_0 - k^{0}_{+2}A^0B^0_2 \Delta H^2_2/RT_0 \\ -k^{0}_{+1}A^0B^0_1 \Delta H^1_1/RT_0 \end{pmatrix}.
\]

The above zero-order equation and the two first-order equations, i.e., three equations, are then used to determine the expressions of three quantities, the thermokinetic parameters of the second step, \(k^{0}_{+2}\) and \(\Delta H^2_2/RT_0\), in terms of the observables \(B^0_1, B^{1\sin}_1,\) and \(B^{1\cos}_1\), the known thermokinetic parameters of the first step \(k^{0}_{+1}\) and \(\Delta H^1_1/RT_0\), and the conserved quantities \(N, N_1,\) and \(N_2\). The expressions of \(k^{0}_{+2}\) and \(\Delta H^2_2/RT_0\) can be considered as functions of the frequency \(\omega\) and are identified to the test functions \(G_2(\omega)\) and \(H_2(\omega)\), respectively. We here only display the first kinetic invariant, \(G_2(\omega)\), the remaining results being contained in the Appendix,

\[
k^{0}_{+2,1} \equiv G_2(\omega) = -\frac{(A^0 - B^0_1 + N_1 - N)(A^0(A^{1\sin} B^0_1\omega + B^{1\cos}_1 N_1) + A^{1\cos} B^0_1 k^{0}_{-1}(N_1 - B^0_1))}{A^0 B^0_1 \left( (A^0)^2 A^{1\cos} + A^0 (B^{1\cos}_1 N_2 - 2 A^{1\cos}(B^0_1 - N_1 + N)) + A^{1\cos}(B^0_1 - N_1 + N) (B^0_1 - N_1 - N_2 + N) \right)}, \tag{17}
\]

with

\[
A^0 = \frac{k^{0}_{-1}(N_1 - B^0_1)}{B^0_1 k^{0}_{+1}}, \tag{19}
\]

\[
A^{1\sin} = \frac{A^0 \left( (B^0_1)^2 \Delta H^1_1 k^{0}_{-1} - B^{0\Delta H^1_1}_1 k^{0}_{-1} N_1 + B^{0\sin}_1 k^{0}_{-1} N_1 \right)}{B^{0\Delta H^1_1}_1 k^{0}_{-1} (B^0_1 - N_1)}, \tag{20}
\]

\[
A^{1\cos} = \frac{A^0 (B^{0\Delta H^1_1}_1 k^{0}_{-1} \sin(\omega) + B^{0\cos}_1 k^{0}_{-1} N_1)}{B^{0\Delta H^1_1}_1 k^{0}_{-1} (B^0_1 - N_1)}. \tag{21}
\]

Once \(k^{0}_{+2}\) has been determined, the value of the rate constant \(k^{0}_{+2}\) is deduced from the expression of the equilibrium constant given in Eq. (12).

The specific aim of the paper is to entirely elucidate two-step mechanisms for which partial information on the first step is supposed to be known. Figure 1 summarizes the whole protocol. The two first phases, E1 and T1, consist in eliminating cases that are not solvable in the typical framework defined by Eqs. (1) and (2) or Eqs. (1) and (3). More precisely, in the experimental phase E1, species A and B1 are mixed without B2. The reaction described by Eq. (1) takes place. When equilibrium is reached, a small temperature modulation is applied and the amplitudes \(B^{1\sin}_1(\omega)\) and \(B^{1\cos}_1(\omega)\) of concentration oscillations of the tagged species are recorded in a wide range
of excitation frequencies. To evaluate the number of relaxation times in the absence of B2, we rely on the test function $G(\omega)$ that we have recently established.\(^{36,37}\)

$$G(\omega) = -\frac{B_1^{1\cos}}{\omega B_1^{1\sin}}. \tag{22}$$

No speculation on the obeyed mechanism is necessary. As found in our previous publications,\(^ {36,37}\) $(n - 1)$ steps or vertical asymptotes of the function $G(\omega)$ correspond to $n$ characteristic times. When $n = 1$, $G(\omega)$ is constant and its value is equal to the single characteristic time. If $G(\omega)$ is not constant, the mechanism possesses two or more characteristic times without $B_2$. It proves that at least one of the species, i.e., $A$, $B_1^1$, or $C_1$, is involved in a chemical reaction other than Eq. (1). It can typically happen in ligand recognition when either an induced fit or a conformational selection mechanism is involved.\(^ {8-11}\) In this case, introduction of $B_2$ would yield at least three steps, a situation out of the scope of the present article.

Once the two first phases of the protocol, E1 and T1, have been successfully passed, the second experimental phase, E2, can be undertaken: species $A$, $B_1^1$, and $B_2$ are mixed for given values of the conserved quantities $N$, $N_1$, and $N_2$; a small temperature modulation is applied; in a large range of frequencies and for the tagged species, the steady state concentration $N^0$ as well as the amplitudes of the concentration oscillations $B_1^{1\sin}$ and $B_1^{1\cos}$ are measured. In order to determine the number of characteristic times in the presence of $B_2$, we again plot the function $G(\omega)$ during phase T2. If $G(\omega)$ is constant, we conclude that $B_2$ does not interact with any species involved in the first step. Then, the mechanism given by Eq. (1) is validated with or without $B_2$. If $G(\omega)$ has more than one step or vertical asymptote, the mechanism with $B_2$ has more than two characteristic times. This case has not been considered in our investigations and still remains to be studied.

The case where $G(\omega)$ has exactly one step or vertical asymptote corresponds to the situations typically addressed in this paper and for which the mechanism can be checked by following the computational phases T3–T6. Phase T3 consists in assuming a mechanism obeying Eqs. (1) and (2) or Eqs. (1) and (3) for given values of the stoichiometric coefficients $(a_1, b_1, c_1, a_2, b_2, c_2)$ or $(a_1, b_1, c_1, a'_2, b'_2, c'_2)$, respectively. Then, similarly to what has been exemplified above for the $(1,1,1,1,1,1)$ sextuplet, in phase T4, the rate equations are used to determine the functions $G_2(\omega)$ and $H_2(\omega)$, which are, respectively, equal to the rate constant $k_{12}^0$ and the enthalpy of reaction $\Delta H_2/RT_0$ of the second step for the assumed mechanism. During phase T5, the graphs of $G_2(\omega)$ and $H_2(\omega)$ are plotted using the known values of $k_{12}^0$ and $\Delta H_2/RT_0$ and the experimental data $N$, $N_1$, $N_2$, $B_1^1$, $B_1^{1\sin}$, and $B_1^{1\cos}$ obtained during phase E2. If either $G_2(\omega)$ or $H_2(\omega)$ is not constant, the assumed mechanism does not coincide with the scrutinized reactive system. As a consequence, the procedure is iterated from phase T3 and another mechanism has to be assumed and
checked. Finally, the procedure is stopped when the graphs of \(G_2(\omega)\) and \(H_2(\omega)\) are both constant. In this case, the assumed mechanism is corroborated, i.e., the six chosen stoichiometric coefficients are confirmed. In addition, the values of the three thermokinetic constants \(k_0^1\), \(k_1^0\), and \(\Delta H_2/(RT_0)\) are determined: \(k_0^0\), and \(\Delta H_2/(RT_0)\) are, respectively, given by the values of \(G_2(\omega)\) and \(H_2(\omega)\) and \(k_0^1\) is deduced from an equation similar to Eq. (12) but obtained for the assumed mechanism under consideration.

IV. PROTOCOL VALIDATION

To simulate an experiment where a mechanistic hypothesis is tested, we need values for the average concentration in the labeled species \(B_j\) as well as for the related first-order amplitudes of oscillations. To this goal, we solve the rate equations associated with different mechanisms. As far as single-step reactions are concerned, in phases E1 and T1, for instance, we rely on our previously published results.\(^{36}\) When two steps are involved, we follow a procedure inverse to the one presented in Sec. III: we express the three concentrations \(B_j^0\), \(B_j^1\), and \(B_j^2\) in terms of the thermokinetic constants \(k_0^1\) and \(\Delta H_2/(RT_0)\), again relying on the formal solver of Mathematica—see the supplementary material.\(^{49}\) Then, a simulated set of responses is drawn by introducing the expressions for \(B_j^0\), \(B_j^1\), and \(B_j^2\) associated with the investigated chemical system in the generic test function \(G(\omega)\) and in the specific test functions \(G_2(\omega)\) and \(H_2(\omega)\) related to the hypothesized mechanism.

Figures 2 and 3 provide simulated \(G(\omega)\) graphs for various chemical systems. During phases E1 and T1, when \(B_2\) is absent, one expects to face a single-step mechanism corresponding to the sole Eq. (1). We indeed obtain flat responses for the \((1,1,1)\) and \((0,1,1)\) reactive systems (see Fig. 2). In contrast, the conformational selection and the induced fit cases yield a step or a vertical asymptote. It is in line with the fact that both are two-step mechanisms associated with Eqs. (1) and (2) for \((1,1,1,0,1)\) and Eqs. (1) and (3) for \((1,1,1,1,0,1')\), respectively. Regarding phases E2 and T2, when \(B_3\) is present, we anticipate all the reactive systems to obey a two-step mechanism. As displayed in Fig. 3, the responses for the various \((a_1,b_1,c_1,a_2,b_2,c_2)\) or \((a_1,b_1,c_1,a'_2,b'_2,c'_2)\) sextuplets actually exhibit a single step or vertical asymptote.

To evaluate the subsequent phases of the protocol, we will focus on a single hypothetical mechanism, the one that corresponds to Eqs. (1) and (2) and to the sextuplet \((1,1,1,1,1,1)\). We plot the graphs of the functions \(G_2(\omega)\) and \(H_2(\omega)\) built for this mechanistic assumption (see Eqs. (17) and (A1)) and for the observables provided by various reactive systems that can be associated with Eqs. (1) and (2) or Eqs. (1) and (3). To study only the influence of the mechanism on \(G_2(\omega)\) and \(H_2(\omega)\), we always use the same values of the thermokinetic parameters.

The graphs of the function \(G_2(\omega)\) obtained for different investigated reactive systems are given in Fig. 4. First of all, most of the values of the functions computed for data \(B_j^0\), \(B_j^1\), and \(B_j^2\) associated with mechanisms different from \((1,1,1,1,1,1)\) are negative. This result is sufficient to refute the hypothesis about the mechanism, since the function \(G_2(\omega)\) is supposed to provide the value of a rate constant. Obtaining negative values of \(G_2(\omega)\) in the entire frequency range leads to the strong presumption that the probed mechanism differs from the assumed one. Then, all the mechanisms that differ from \((1,1,1,1,1,1)\) lead to steps or vertical asymptotes, proving the
The function \( G(\omega) \) is assumed to obey \((a_1, b_1, c_1, a_2, b_2, c_2) = (1, 1, 1, 1, 1, 1)\). The color lines correspond to the responses simulated for chemical systems associated with various stoichiometric coefficients \((a_1, b_1, c_1, a_2, b_2, c_2)\) in Eqs. (1) and (2) or \((a_1, b_1, c_1, a_2', b_2', c_2')\) in Eqs. (1) and (3). The values for the different kinetic parameters are identical to the ones used in Fig. 2. The function \( G(\omega) \) has the same unit as \( k_0^B \); the unit of \( \omega \) is \( s^{-1} \).

The nonlinearities of the rate equations given in Eqs. (9) and (10) are certainly related to the discriminating power of the function \( G(\omega) \) built for the \((1, 1, 1, 1, 1, 1)\) mechanism. Figure 5 provides the graphs of the function \( H(\omega) \). When the probed mechanism is associated with the stoichiometric coefficients \((1, 1, 1', 1', 1')\) or \((1, 1, 1', 0', 1')\), \( H(\omega) \) shows small variations that would only become apparent for well-resolved experimental data. In fact, enthalpies of reaction are supposed to vary over two orders of magnitude at most, against about ten orders for rate constants; accordingly, a function based on an enthalpy of reaction is expected to be less discriminative than a function based on a rate constant. However, it is always safer to plot both \( G(\omega) \) and \( H(\omega) \) to check a mechanism.

In order to specify the limitations of the technique, we evaluate the effects of the experimental noise inherently present during the measurement of \( B_0^1 \), \( B_1^1 \), and \( B_1^{1\sin} \). More precisely, we scrutinize its impact on the aspect of \( G(\omega) \), the \textit{a priori} best discriminating function. Once again, we choose the function given in Eq. (17), which corresponds to the mechanism described in Eqs. (1) and (2) and the \((1, 1, 1, 1, 1, 1)\) stoichiometry. We also assume that a same relative uncertainty, \( \Delta B_0^1 / B_0^1 = \Delta B_1^1 \cos / B_1^1 \cos = \Delta B_1^{1\sin} / B_1^{1\sin} \), affects all three measurements. Then, for a regular, log-scale sampling of 10 \( \omega \) values per decade, we numerically determine the extremal values, \( G_{\min}(\omega) \) and \( G_{\max}(\omega) \), of the function \( G(\omega) \) when the data \( B_0^1 \), \( B_1^1 \cos \), and \( B_1^{1\sin} \) vary within an interval associated with a given percentage of uncertainty. Results are displayed in Fig. 6. The domains bounded by the two \( G_{\min}(\omega) \) and \( G_{\max}(\omega) \) lines are represented in increasingly clear gray levels, from dark gray for 5% of uncertainty to light gray for 25%. A value of \( G(\omega) \) computed from experimental measurements spoiled by a noise of a given amplitude is thus supposed to fall into a given domain. The most striking result is that noise associated with \((1, 1, 1, 1, 1, 1)\) induces a similar behavior as the exact values of \( B_0^1 \), \( B_1^1 \cos \), and \( B_1^{1\sin} \) computed for mechanisms different from \((1, 1, 1, 1, 1, 1)\). In particular, noise leads to scattered points lying between vertical asymptotes which are located around the frequencies characteristic of the singularities observed in the graphs of the \( G_2 \) functions obtained for \((1, 1, 1', 0', 1'), (1, 1, 1', 1', 1'), \) and \((0, 1, 1, 1', 1', 1')\). However, as mentioned above, the effect of noise does not prevent from deducing a reliable conclusion when the flat behavior


FIG. 6. Evaluation of the influence of noise in the measurements of $B_1^0$, $B_1^{1\text{cos}}$, and $B_1^{1\text{sin}}$ on the $G_2(\omega)$ function built when the mechanism is assumed to obey Eqs. (1) and (2) with $(a_1,b_1,c_1,a_2,b_2,c_2) = (1,1,1,1,1,1)$. The values for the different thermokinetic parameters are identical to the ones used in Fig. 2. Domains in which should fall noisy values of $G_2(\omega)$ computed from acquisitions associated with increasing percentages of inaccuracy are displayed using gray levels, from $\Delta B_1^0/B_1^0 = \Delta B_1^{1\text{cos}}/B_1^{1\text{cos}} = \Delta B_1^{1\text{sin}}/B_1^{1\text{sin}} = 5\%$ (dark gray) to $25\%$ (light gray). The $G_2(\omega)$ function has the same unit as $k_{s_2}^0$; the unit of $\omega$ is s$^{-1}$. The color lines are the same as in Fig. 4.

at low frequency in the graphs of $G_2(\omega)$ is associated with a negative limit. Indeed, even for a level of inaccuracy of the order of $25\%$, the plot of the test functions that would be determined for the $(1,1,1,1',0',1')$, $(1,1,1,1',1',1')$, and $(1,1,1,0,1,1)$ mechanisms would yield a cloud around a negative mean value, allowing for the rebuttal of the hypothesis with a probability close to 1. Moreover, the $\Delta k_{s_2}^0 = 12$ from the one of $(1,1,1,1,1)$ that would be obtained for $k_{s_2}^{\text{app}} = 33$. Using Eq. (12) for the equilibrium constant and the same values of the observables, $A_0$, $B_1^0$, and conserved quantities, $N$, $N_1$, and $N_2$, we deduce that $k_{s_2}^{\text{app}} = 16.5$. The other thermokinetic parameters, related to the first step, are supposed to be known and equal to the ones of Fig. 2 caption.

Among the mechanisms checked in Fig. 6, only the $(0,1,1,1',1',1')$ case results in a positive low frequency limit. Therefore, its behavior will be difficult to distinguish from the one associated with $(1,1,1,1,1,1)$: in both cases, one should obtain a flat cloud of points around a positive mean value at low frequency that spreads with vertical asymptotes at high frequency. A more detailed analysis is consequently required. Starting from the data collected at low frequency, one can first compute their average and restate the test: we have to distinguish the behavior of $(0,1,1,1',1')$ that would be obtained for $k_{s_2}^0 = 12$ from the one of $(1,1,1,1,1)$ that would be obtained for $k_{s_2}^{\text{app}} = 33$. Using Eq. (12) for the equilibrium constant and the same values of the observables, $A_0$, $B_1^0$, and conserved quantities, $N$, $N_1$, and $N_2$, we deduce that $k_{s_2}^{\text{app}} = 16.5$. The other thermokinetic parameters, related to the first step, are supposed to be known and equal to the ones of Fig. 2 caption.

We subsequently consider the function $G_2(\omega)$ given in Eq. (17) for the stoichiometry $(1,1,1,1,1,1)$ and for $k_{s_2}^{\text{app}}$. Again, we determine the two lines $G_{2\text{min}}(\omega)$ and $G_{2\text{max}}(\omega)$ which bound the domain of uncertainty of $G_2(\omega)$ when assuming that $\Delta B_1^0/B_1^0$, $\Delta B_1^{1\text{cos}}/B_1^{1\text{cos}}$, and $\Delta B_1^{1\text{sin}}/B_1^{1\text{sin}}$ are equal to a given percentage (gray areas in Fig. 7(a)). In parallel, we sample the considered frequency interval with 28 equally spaced $\omega$ points (in decimal logarithmic units) and compute the corresponding 28 $G_2(\omega)$ values relying on the $B_1^0$, $B_1^{1\text{cos}}$, and $B_1^{1\text{sin}}$ associated with the $(0,1,1,1',1',1')$ stoichiometry (red triangles in Fig. 7(a)). Finally, in Fig. 7(b), we define a probability $p$ as the normalized number of sampled $G_2(\omega)$ values found outside a given
uncertainty domain. The values of $p$ only give an indication of the chances of success of the method, since we admit that noise only affects the expected results for the assumed $(1,1,1,1,1)$ mechanism and not what would be measurements for the true $(0,1,1,1',1',1')$ mechanism. Furthermore, discrimination can surely be optimized by a more careful choice of the sampling interval and density, or by exclusively selecting frequencies close to the vertical asymptote.

A complementary way to further validate the protocol requires reaction networks whose kinetics has been studied in detail and for which the rate constants and activation energies are known for different ligands. Orthosteric competitive ligand binding on carbonic anhydrase II offers this possibility.\(^{65}\) We choose to apply the procedure to the competition between $B_1 = 1,3$-benzenedisulfonamide $(1,3$-BDS) and a second ligand $B_2$ which can be $4$-carboxybenzenedisulfonamide $(4$-CBS) or sulfanilamide. The question is to determine if the system actually follows the assumed mechanism described in Eqs. (1) and (2) for the $(1,1,1,1,1)$ stoichiometry or if it complies with some other dynamics, involving either two binding sites, i.e., Eqs. (1) and (2) with $(1,1,1,1,2,1)$ or allostery, i.e., Eqs. (1) and (3) with $(1,1,1,1',1',1')$. Again, Eq. (17) for the $G_2(\omega)$ function is used and the graphs of $G_2(\omega)$ are obtained by computing the values of $B^0_1$, $B^{\text{con}}_1$, and $B^{\text{sin}}_1$ for the three mechanisms $(1,1,1,1,1), (1,1,1,1,2,1)$, and $(1,1,1,1',1',1')$, relying on experimental values of the thermokinetic parameters.\(^{65}\) Results are given in Fig. 8(a) for the competition between $B_1 = 1,3$-BDS and $B_2 = 4$-CBS and in Fig. 8(b) for the competition between $B_1 = 1,3$-BDS and $B_2 = $ sulfanilamide.

The two ligands of Fig. 8(a) have close equilibrium binding constants, $K_1 = 4.2 \times 10^{-7}$ M and $K_2 = 6.2 \times 10^{-7}$ M, and we found that choosing $N_i = K_i$ for $i = 1,2$ and $N_i < N_j$ ensures favorable conditions for this competitive assay. Indeed, the values of the function $G_2(\omega)$ obtained for the two mechanisms $(1,1,1,1',1')$ and $(1,1,1,2,1)$ not only lead to vertical asymptotes at high frequency, which may be misleading in the presence of noisy measurements, but also to flat behaviors associated with truly negative limits at low frequency (up to 20 times larger than $k^0_2$ in absolute value). Hence, the graphs of $G_2(\omega)$ allow us to set aside the two mechanisms $(1,1,1,1',1')$ and $(1,1,1,2,1)$ and to support the $(1,1,1,1,1)$ hypothesis without ambiguity. The results given in Fig. 8(a) can be considered as a successful test of the method in the case of experimental thermokinetic values.

In contrast, the example treated in Fig. 8(b) illustrates some limitations of the method. When the two ligands have equilibrium binding constants that differ by more than one order of magnitude, $K_1 = 4.2 \times 10^{-7}$ M and $K_2 = 2.7 \times 10^{-6}$ M, it is more difficult to distinguish the different mechanisms. As observed in Fig. 8(b) at low frequency, the mechanism associated with $(1,1,1,2,1)$ leads to the same constant limit as $(1,1,1,1,1)$ and cannot be rebutted. Less problematic but nevertheless annoying, the $(1,1,1,1',1',1')$ mechanism yields a positive horizontal limit at low frequency and a vertical asymptote at high frequency, in a way similar to the $(0,1,1,1',1',1')$ mechanism in Figs. 6 and 7. In this case, the effect of noise in the measurement of $B^0_1, B^{\text{con}}_1$, and $B^{\text{sin}}_1$ could make a definitive conclusion delicate.

V. DISCUSSION

The application conditions of the protocol are not restrictive and many biological mechanisms of interest fall within the present framework. Binding of one or two ligands to proteins

![Figure 8](https://example.com/figure8.png)

**Fig. 8.** Frequency behavior of the $G_2(\omega)$ function built to validate a mechanism of orthosteric competitive binding on carbonic anhydrase II, i.e., $(a, b, c, a_2, b_2, c_2) = (1,1,1,1,1)$ at $T = 18\, ^\circ\mathrm{C}$. Units of $G_2$ are $\text{M}^{-1}$ s$^{-1}$ and the ones of $\omega$ are $\text{s}^{-1}$. In both cases, the tracer is assumed to be $B_1 = 1,3$-benzenedisulfonamide $(1,3$-BDS), for which $k^0_2 = 1.4 \times 10^{10}$ s$^{-1}$, $k^0_1 = 0.059$ s$^{-1}$, and $\Delta H_1/(RT_0) = 10.4$. The blue dashed lines correspond to the responses simulated for the stoichiometry $(1,1,1,1',1')$ and the purple dotted ones for $(1,1,1,2,1)$. (a) Left: the second ligand is $B_2 = 4$-carboxybenzenedisulfonamide $(4$-CBS), for which $k^0_2 = 2.6 \times 10^{10}$ s$^{-1}$, $k^0_1 = 0.016$ s$^{-1}$, and $\Delta H_2/(RT_0) = -18.9$. The concentrations are selected such that $N = 5 \times 10^{-6}$ M, $N_1 = 4.2 \times 10^{-7}$ M, and $N_2 = 6.2 \times 10^{-7}$ M. (b) Right: the second ligand is $B_2 = $ sulfanilamide, for which $k^0_2 = 1.9 \times 10^{10}$ s$^{-1}$, $k^0_1 = 0.052$ s$^{-1}$, and $\Delta H_2/(RT_0) = -14.9$. The concentrations are selected such that $N = 10^{-6}$ M, $N_1 = 4.2 \times 10^{-7}$ M, and $N_2 = 2.7 \times 10^{-6}$ M.
with possible conformational changes, before or after fixation, can most of the time be elucidated using the proposed procedure. As discussed below, only the mechanism of induced fit cannot be checked. In addition, we have given examples where our method could be adapted to the design of drugs, whether they act as competitive or uncompetitive inhibitors, whether they target a single macromolecule or a protein-protein interaction. Finally, an implementation in vivo cannot be excluded since the technique is compatible with more realistic far-from-equilibrium conditions and since it has been demonstrated recently that concentration oscillations upon thermal harmonic forcing can be collected in living cells.39

The validation of induced fit and conformational selection through the detection of a single tagged species raises specific problems because the idea consisting in characterizing a first step without B2 may lose its immediate meaning. The possibility to apply the present protocol to discriminate between the two mechanisms thus has to be reexamined. In the case of induced fit, the reaction is always associated with the (1,1,1,1,0,1) sextuplet, whether we observe the protein or the ligand. Consequently, the second step cannot be avoided when trying to characterize the first one and it seems impossible to determine the values of the thermokinetic constants $k_{2a1}$ and $\Delta H_1/(RT_0)$ by detecting only species $B_1^*$. To completely elucidate such a mechanism, we suggest to implement the procedure that we proposed for general two-step mechanisms and which requires two tagged species.37 Similarly, when the tagged species $B_1^*$ is the ligand, conformational selection is associated with the sextuplet (1,1,1,1,0,1) so that nothing prevents the second step to occur when studying the first one. Nevertheless, if the enzyme can be tagged to play the role of species $B_1^*$ conformational selection can be viewed as $(0,1,1,1,1,1')$. The first reaction can then be studied without $B_2$, $k_{2a1}$ as well as $\Delta H_1/(RT_0)$ determined, and the Fig. 1 protocol applied up to the end. To summarize, although some reactions cannot be asserted relying on the strategy described in the present paper, opportunities to apply the proposed protocol are numerous if the experiment is carefully devised, if the scrutinized species is appropriately chosen. Some adaptation may be required, including that the tag is carried by a species other than $B_1$, for example, by A or C1.

An other feature of the method is that the technique employed to measure the rate constants and the enthalpy of the first reaction does not need to include checking of the corresponding mechanism. The stoichiometric coefficients $(a_1,b_1,c_1)$ do not have to be ascertained and the existence of other hidden steps is not precluded. Although the information on probe reaction (1) is used to elucidate the step of interest, i.e., to determine the stoichiometric coefficients and the thermokinetic parameters of Eq. (2) or (3), the whole procedure remains consistent since its last phases also constitute a validation of the hypotheses related to the first step.

Eventually, we propose to evaluate how the proposed protocol can be implemented in the laboratory. Most of the setups that have been used for modulating the temperature of a reactive system rely on laser38,39,40 or resistive heating40 when millisecond kinetics need to be investigated. However, for events occurring on the second time scale, water baths can provide a satisfactory alternative.61,62 As a consequence, a window of up to six orders of magnitude, from nearly $10^{-3}$ to $10^3$ Hz, is opened to the experimentalists. Of course, the validation of any mechanism will only be effective in this interval, since we expect significant deviation at higher frequencies, corresponding to more elementary steps, that involve smaller temporal scales. Nevertheless, the accessible range of rate constants is today appropriate to investigate phenomena such as ligand binding or protein-protein interaction.

As far as uncertainties are scrutinized, the effect of noise in concentration measurements is certainly the main source of concern. Indeed, modern function generators used for Joule heating produce currents with less than 1% of error. Furthermore, temperature deviation can be corrected38 with the help of molecular thermometers36–38 dissolved in solution. Typically, thermal forcing is applied with a few degree amplitude,38–41 which enables to apply a perturbative approach, to remain in a domain where the chemical system responds linearly to the temperature change while enough signal can be collected and concentration oscillations detected. The most sensitive instruments are built around an epifluorescence microscope and reactive systems are diluted at several micromoles. From the data displayed in the literature, the experimental noise can be evaluated to be around 10%, a value that can be lowered if one uses photomultiplier tubes and lock-in amplifiers70 instead of CCD cameras and numerical demodulation40,41.

In the last part of the protocol evaluation process, we thus have introduced a noise in concentration determination which amplitude is in a realistic range. We then realized that these uncertainties tend to induce the same kind of accidents in the test functions as those due to observables associated with other mechanisms. Nevertheless, we have checked that, in many cases, negative values of the test function derived from the expression of a rate constant were obtained when the mechanism was differing from the assumed one, leading without ambiguity to the rebuttal of the hypothesis. Furthermore, to deal with unclear situations, we could sketch a procedure able to assess some confidence level.

**VI. CONCLUSIONS**

In this paper, we extended the scope of a method that we recently proposed to check simple chemical mechanisms and to determine the full set of associated rate constants and enthalpies of reaction.36,37 The novel protocol discussed here involves the detection of a single chemical species to ascertain usual types of two-step mechanisms. Its application simply requires that a same species, not necessarily the tagged one, intervenes in the two steps and that the thermokinetic parameters of the first step could have been determined independently.

According to the general methodology that we previously devised,36,37 we rely on the measurement of concentration modulations obtained in response to some thermal harmonic forcing of small amplitude.38–41 We assume a chemical mechanism and build test functions of the excitation frequency whose graph can be plotted from the knowledge of the thermokinetic parameters of the first step and of the concentration oscillations of the tagged species. By construction, these test functions are constant if the oscillation amplitudes used to evaluate them obey the laws of kinetics associated with the assumed
mechanism. Reciprocally, we find non constant graphs for at least one of the test functions when using concentration oscillations associated with mechanisms different from the assumed one. As a consequence, the test functions can be harnessed to check which mechanism is obeyed by the chemical system. One simply needs to determine their behavior using experimental data for the concentration oscillations: the hypothesis about the mechanism is confirmed if all the test functions are constant in a wide range of excitation frequencies.

ACKNOWLEDGMENTS

This work was supported by the T-KiNet grant from the French national research agency (ANR), the T-DropTwo grant from the Labex NanoSaclay, the T-ProtUV grant from the Cnano IdF, and the Convergence program of the Université Pierre et Marie Curie. We thank D.-S. Lee-Taupin for help in preparation of the figures.

APPENDIX: TEST FUNCTION DEDUCED FROM THE ENTHALPY OF REACTION

When the mechanism given in Eqs. (1) and (2) is assumed for the stoichiometric coefficients (1, 1, 1, 1, 1), the enthalpy of the second reaction, $\Delta H_2/(RT_0)$, can be expressed in terms of the observables according to the following Eqs. (A1) and (A2), which are used to define the test function $H_2(\omega)$:

\[
\frac{\Delta H_2}{RT_0} = H_2(\omega) \equiv \left\{ \begin{array}{l}
\left. - (A^0)^2 \right. \\
+ k_1^{-1} \left( A^1 \cos(B_0^1) - A^1 \sin(B_0^1) \right) \\
+ A^0 \cos k_1^{-1} \left( N(2B_1^0 - 2N_1 - 2N_2) \right) \\
+ A^1 \sin k_1^{-1} \left( N(2B_1^0 - 2N_1 + N_2) - N_1 - N_2 + N \right) \\
+ B_1^1 \cos k_1^{-1} \left( N(2B_1^0 - 2N_1 + N_2) - N_1 - N_2 + N \right) \\
\end{array} \right. \\
D \equiv (A^0 - B_0^1 + N_1 - N)(A^0 - B_0^1 + N_1 + N_2 - N) \left( (A^1 \cos B_0^1) + A^1 \sin B_0^1 \right) \left( (A^1 \cos k_1^{-1} N_1) + A^1 \sin k_1^{-1} N_1 \right) \left( (A^1 \cos B_0^1) + A^1 \sin B_0^1 \right) \left( (A^1 \cos k_1^{-1} N_1) + A^1 \sin k_1^{-1} N_1 \right)
\]

and with $A^0, A^1 \cos$, and $A^1 \sin$ given in Eqs. (19)–(21).
