Journal of Mathematical Chemistry Vol. 35, No. 3, March 2004 (© 2004)

Mechanism equivalence in enzyme–substrate reactions: Distributed differential delay in enzyme kinetics

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Received 4 November 2003; revised 11 March 2004

We consider single enzyme–substrate reaction mechanisms involving multiple complexes and demonstrate that these are equivalent to a distributed delay system without complexes. The distribution of the delay is determined by the number of intermediates and the relative sizes of the rates of the individual reaction mechanisms. We also consider the limit where there are a large number of intermediate complexes, and the conditions under which a number of known reaction mechanisms are equivalent. The present formalism brings forth new perspectives in the implementation of experimental techniques to rule out particular reaction mechanisms by studying the distribution of the delay between reactant mixing and product formation.

KEY WORDS: enzyme kinetics, reaction intermediates, distributed differential delay, distinguishable, equivalent mechanisms

AMS subject classification: 92C45, 80A30, 34K17, 34K60, 34K99

1. Introduction

In the 19th century, the first scientists studying enzyme kinetics of the single enzyme–substrate reaction experienced a number of difficulties. The experimental practice was to follow the reaction over an extended period of time, and to explain observations in terms of the solutions of second-order rate equations used in chemical kinetics. But then, the British chemist Brown [1] found that the rate of enzyme-catalysed reactions deviated from second-order kinetics. In 1902, Brown [2] proposed the existence of an enzyme–substrate complex in a purely

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0259-9791/04/0300-0253/0

kinetics context with a fixed lifetime to form the product. This was the first time that the existence of the enzyme–substrate complex was proposed in an enzymatic reaction. Brown's model was criticised [3,4] for being intuitive and lacking mathematical foundation. Brown's chemical mechanism consists of a reaction between an enzyme E and a substrate S, forming the enzyme–substrate complex $E \cdot S$, which yields with a delay the product P:

$$\mathrm{S} + \mathrm{E} \xrightarrow{\mathrm{k}} \mathrm{E} \cdot \mathrm{S} \xrightarrow{\mathrm{delay}} \mathrm{E} + \mathrm{P}. \tag{1}$$

Historically, this criticism is understandable as systems with delays were first studied in various disciplines during the years 1920–1940 [5], led by the pioneer work of Sharpe and Lotka [6] and Volterra [7] in epidemiology and ecology. Brown's model of enzyme action was succeeded by the Henri [8–10] mechanism which follows a mass-action kinetics. This model is conventionally attributed to Michaelis and Menten [11] although these authors clearly recognised Henri as the originator.

Brown could be credited with formulating the essential ideas of the Henri–Michaelis–Menten mechanism of enzyme action [12]. We can also acknowledge him as the first biochemist to propose a delayed effect in chemical kinetics. His contribution should not be underestimated, because there is nowadays a great interest in the application of delayed differential equations (DDEs) for studying model reduction in chemistry [13, and references therein] and in gene transcription regulation [14–16, and references therein]. Moreover, a mathematical formulation of Brown's model has recently been developed to study the chemical acceptability of delayed-mass action models [17].

In modelling a chemical system, it is sometimes necessary to take into account the time delays inherent to the system under consideration. On the other hand, the inclusion of the delay is more often introduced to simplify the mathematical description of a kinetic model or because there are details in the reaction mechanism which are unknown. As far as we know, Ninio [18] was the first biochemist to construct a delayed enzyme–substrate reaction by sequences of conventional elementary chemical steps involving the non-allosteric binding of several substrates to a multimeric enzyme. This is nowadays a well-known result: an irreversible linear chain of reactions:

$$\xrightarrow{k_0} \mathrm{C}_1 \xrightarrow{k_1} \mathrm{C}_2 \xrightarrow{k_2} \cdots \xrightarrow{k_{n-1}} \mathrm{C}_n \xrightarrow{k_n}, \tag{2}$$

in which all the rate constants are the same ($k_i = k$) can be described by a differential delay equation with a γ-distributed delay [5,13,19,20].

One of the major difficulties in the study of a reaction mechanism evolves around the modelling of chemical intermediates. They are generally in low concentrations at the reaction media and their lifetime is very brief [21]. Therefore, their existence is often inferred rather than observed. For this reason, there is a tradition in chemistry to propose a reasonable minimal model in which it is

necessary to include some unobserved intermediate [22]. Consider the reversible enzyme–substrate Michaelis–Menten reaction:

$$\mathrm{S} + \mathrm{E} \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E} + \mathrm{P}, \tag{3}$$

where k_i are positive rate constants. To obtain a reasonable minimal model of a real enzymic reaction biochemists have postulated an enzyme–product complex arising from the enzyme–substrate complex by an intramolecular reaction, which constitutes the essential catalytic step, e.g. [23, 24]. The simplest possible overall mechanism is then

$$\mathrm{S} + \mathrm{E} \underset{k_{-0}}{\overset{k_0}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{P} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E} + \mathrm{P}. \tag{4}$$

In this reaction, transition state activated complexes are involved at each reversible reaction step. However, the latter reaction is indistinguishable kinetically from the simpler scheme (3) under quasi-steady-state studies [4]. Reactions (3) and (4) would also be indistinguishable kinetically in quasi-steady-state studies from the Van Slyke–Cullen reaction mechanism [25]:

$$\mathrm{S} + \mathrm{E} \overset{k_0}{\rightarrow} \mathrm{E}\cdot\mathrm{S} \overset{k_1}{\rightarrow} \mathrm{E} + \mathrm{P}, \tag{5}$$

if k_{-i} are small and the initial product concentration is zero.

To reinforce these statements on distinguishable and equivalent reactions we should call attention to Cleland's dictum that nothing which takes place within a "central complex" (equivalent to the enzyme–substrate complex) can possibly affect the rate law [12]. Moreover, it has been shown in a number of chemical reactions [17, 22, 26] that we can replace an intermediate by a delay term preserving the dynamical behaviour of the model.

In the case of enzyme catalysed reactions, the intermediate steps between the reactants and products are the most interesting and important to understand the process of catalysis [21]. A number of experimental techniques such as rapid mixing, sampling techniques, flash photolysis and relaxation methods [27] have been developed to study in detail these events. However, the kinetics aspects of distinguishable or equivalent enzyme kinetics mechanisms in our opinion remain inadequately understood and analysed. Particularly the criteria require to distinguish mechanisms with various enzyme–substrate complexes or intermediates. In this paper, we consider the single enzyme–substrate reaction mechanism originally proposed by Van Slyke and Cullen [25] involving multiple and fast enzyme–substrate complexes. We derive criteria for the equivalence of mechanisms with multiple intermediates employing distributed delay systems. We pay special attention to how the delay is related to the reaction parameters.

2. Enzyme–substrate reaction with one complex intermediate

To set the stage, consider a simple reaction between a substrate (S) and enzyme (E) forming irreversibly a enzyme–substrate intermediate complex (C_1), which yields a product (P) plus enzyme. Schematically this reaction is represented by

$$\mathrm{S} + \mathrm{E} \xrightarrow{k_0} \mathrm{C}_1 \xrightarrow{k_1} \mathrm{E} + \mathrm{P}. \tag{6}$$

This reaction can be modelled by a simple set of ordinary differential equations (ODEs). Define S as the concentration of substrate, E as the concentration of enzyme, C_1 as the concentration of complex and P as the concentration of product. Then

$$\begin{aligned} \frac{\mathrm{d}S}{\mathrm{d}t} &= -r(S(t), E(t), t), \\ \frac{\mathrm{d}E}{\mathrm{d}t} &= -r(S(t), E(t), t) + k_1 C_1, \\ \frac{\mathrm{d}C_1}{\mathrm{d}t} &= r(S(t), E(t), t) - k_1 C_1, \\ \frac{\mathrm{d}P}{\mathrm{d}t} &= k_1 C_1. \end{aligned} \tag{7}$$

Here $r(S(t), E(t), t)$ is the reaction term. For notational convenience, define $R(t) = r(S(t), E(t), t)$. The aim of the analysis is to write the equation for P in the form of a distributed delay equation:

$$\frac{\mathrm{d}P}{\mathrm{d}t} = \int_0^\infty f_1(z) R(t - z) \mathrm{d}z, \tag{8}$$

where $f_1(z)$ is the distribution of the delay. The ODE for the complex (7) can be integrated from $-\infty$ to t using an integrating factor:

$$\left[C_1 \mathrm{e}^{k_1 t'}\right]_{-\infty}^{t} = \int_{-\infty}^{t} \mathrm{e}^{k_1 t'} R(t') \mathrm{d}t'. \tag{9}$$

The left-hand side (LHS) evaluated at $-\infty$ vanishes. Introducing the new variable $z = t - t'$ and cancelling a factor of $\mathrm{e}^{k_1 t}$, we have

$$k_1 C_1 = \int_0^\infty k_1 \mathrm{e}^{-k_1 z} R(t - z) \mathrm{d}z. \tag{10}$$

Substituting this into the ODE for the product (7) yields a distributed delay equation (8), where the delay is exponentially distributed:

$$f_1(z) = k_1 \mathrm{e}^{-k_1 z}. \tag{11}$$

3. Enzyme–substrate reaction with two complex intermediates

We now consider the case where there are two intermediate complexes C_1 and C_2. Schematically this reaction can be represented by

$$S + E \overset{k_0}{\rightarrow} C_1 \overset{k_1}{\rightarrow} C_2 \overset{k_2}{\rightarrow} E + P. \tag{12}$$

This reaction can be modelled by a simple set of ODEs

$$\begin{aligned} \frac{dS}{dt} &= -R(t), \\ \frac{dE}{dt} &= -R(t) + k_2 C_2, \\ \frac{dC_1}{dt} &= R(t) - k_1 C_1, \\ \frac{dC_2}{dt} &= k_1 C_1 - k_2 C_2, \\ \frac{dP}{dt} &= k_2 C_2. \end{aligned} \tag{13}$$

The equation for C_1 can be integrated and is given by equation (10). Inserting this into the ODE for C_2 and integrating from $-\infty$ to t using an integrating factor, we obtain:

$$\left[C_2 e^{k_2 t'}\right]_{-\infty}^{t} = \int_{-\infty}^{t} e^{k_2 t'} \int_0^{\infty} k_1 e^{-k_1 z} R(t' - z) dz \, dt'. \tag{14}$$

The LHS evaluated at $-\infty$ vanishes. Introducing the new variable $v_1 = t' - t + z$ and $v_2 = z$, then cancelling a factor of $e^{k_2 t}$ yields

$$k_2 C_2 = \int_{v_1=0}^{\infty} \int_{v_2=0}^{v_1} k_1 k_2 e^{-k_2 v_1} e^{-(k_1 - k_2) v_2} R(t - v_1) dv_2 \, dv_1. \tag{15}$$

Integrating with respect to v_2 gives

$$k_2 C_2 = \int_{v_1=0}^{\infty} k_1 k_2 e^{-k_2 v_1} \frac{e^{-(k_1 - k_2) v_1} - 1}{k_2 - k_1} R(t - v_1) dv_1. \tag{16}$$

Substituting this into the ODE for the product (13) yields a distributed delay equation (8), where the distribution of the delay is

$$f_2(z) = k_1 k_2 \left(\frac{e^{-k_1 z}}{k_2 - k_1} + \frac{e^{-k_2 z}}{k_1 - k_2} \right). \tag{17}$$

Note that if the rate constants k_1 and k_2 differ significantly in magnitude, further simplifications can be carried out.

4. Enzyme–substrate reaction with N-intermediates

We now consider the case where there are N intermediate complexes C_i, where $i = 1, \ldots, N$. This reaction can be modelled by a simple set of ODEs:

$$\begin{aligned}
\frac{dS}{dt} &= -R(t), \\
\frac{dE}{dt} &= -R(t) + k_N C_N, \\
\frac{dC_1}{dt} &= R(t) - k_1 C_1, \\
\frac{dC_i}{dt} &= k_{i-1} C_{i-1} - k_i C_i, \qquad \text{when } i = 2, \ldots, N \\
\frac{dP}{dt} &= k_N C_N,
\end{aligned} \tag{18}$$

where $k_i \neq k_j$ when $i \neq j$. We now show that this system is equivalent to a distributed delay system.

Proposition 1. The N-step reaction process (18) is equivalent to a distributed delay system with

$$f_N(z) = \sum_{i=1}^{N} k_i e^{-k_i z} \prod_{j=1, j\neq i}^{N} \frac{k_j}{k_j - k_i}, \tag{19}$$

where $k_i \neq k_j$ and $N \geq 2$.

This is proved by induction. Assume true for $N = m$. Then

$$\frac{dC_{m+1}}{dt} = \int_0^\infty \sum_{i=1}^{m} k_i e^{-k_i z} \prod_{j=1, j\neq i}^{m} \frac{k_j}{k_j - k_i} R(t - z) dz - k_{m+1} C_{m+1}. \tag{20}$$

This equation can be integrated from $t' = -\infty$ to t using an integrating factor

$$\left[C_{m+1} e^{k_{m+1} t'}\right]_{-\infty}^{t} = \int_{-\infty}^{t} e^{k_2 t'} \int_0^\infty \sum_{i=1}^{m} k_i e^{-k_i z} \prod_{j=1, j\neq i}^{m} \frac{k_j}{k_j - k_i} R(t' - z) dz \, dt'. \tag{21}$$

Introducing the change of variable $v_1 = t - t' - z$ and $v_2 = z$, cancelling a factor of $e^{k_{m+1} t}$, and reversing the order of the finite sum and integration, yields

$$C_{m+1} = \sum_{i=1}^{m} k_i \prod_{j=1, j\neq i}^{m} \frac{k_j}{k_j - k_i} \int_{v_1=0}^{\infty} \int_{v_2=0}^{v_1} e^{-(k_i - k_{m+1}) v_2 - k_{m+1} v_1} R(t - v_1) \, dv_2 \, dv_1. \tag{22}$$

Integrating, we obtain:

$$k_{m+1}C_{m+1} = \int_0^\infty \left\{ \sum_{i=1}^{m} \left(\mathrm{e}^{-k_i v_1} - \mathrm{e}^{-k_{m+1} v_1} \right) k_i \prod_{j=1, j\neq i}^{m+1} \frac{k_j}{k_j - k_i} \right\} R(t - v_1)\, \mathrm{d}v_1. \quad (23)$$

Next substituting the identity

$$\sum_{i=1}^{m+1} k_i \prod_{j=1, j\neq i}^{m+1} \frac{k_j}{k_j - k_i} = \sum_{i=1}^{m} k_i \prod_{j=1, j\neq i}^{m+1} \frac{k_j}{k_j - k_i} + k_{m+1} \prod_{j=1}^{m} \frac{k_j}{k_j - k_{m+1}} = 0, \quad (24)$$

[which is proved in the appendix] into equation (23) gives

$$k_{m+1}C_{m+1} = \int_{z=0}^{\infty} f_{m+1}(z) R(t - z)\, \mathrm{d}z. \quad (25)$$

Therefore by induction the distribution $f_n(z)$ equation (19) is true for all $N \geq 2$.

5. The large-N intermediate limits

The final part of the analysis is to consider the limit of a large number of intermediate complexes. The distribution function f_N can be simplified and the entire reaction sequence can be represented by a simple mechanism (i.e., standard law of mass action kinetics or differential delay system). Consider the Laplace transform (i.e., moment generating function) of the distribution function $f_N(z)$

$$\tilde{f}_N(s) = \int_0^\infty f_N(z) \mathrm{e}^{-sz} dz = \sum_{i=1}^{N} \frac{k_i}{k_i + s} \prod_{j=1, j\neq i}^{N} \frac{k_j}{k_j - k_i}. \quad (26)$$

We consider the case where all reactions are rapid with the exception of one 'rate limiting' step or bottleneck intermediate [20]. Since equation (19) is invariant under reordering k_i, we can set the first step to be the 'rate limiting' step without any loss of generality. Set $k_1 = 1/\tau_r$, where τ_r is $O(1)$, and $k_i = Nb_i$ for $i \geq 2$, where $1/b_i$ is $O(1)$. Define

$$\bar{\tau} = \sum_{i=2}^{N} \frac{1}{k_i} = \frac{1}{N} \sum_{i=2}^{N} \frac{1}{b_i}. \quad (27)$$

τ_r is the reaction time or timescale of the rate limiting step, and $\bar{\tau}$ is the total reaction time of the other $N - 1$ steps. Note that $\bar{\tau}$ can be the same size as τ_r.

The relative size of $\bar{\tau}$ and τ_r determines which simplified model approximates the N-step reaction process. Rewrite equation (26) separating out terms involving k_1:

$$\tilde{f}_N(s) = \frac{1}{1+\tau_r s}\prod_{j=2}^{N}\frac{Nb_j}{Nb_j - 1/\tau_r} + \sum_{i=2}^{N}\frac{Nb_i}{(Nb_i+s)}\frac{1/\tau_r}{(1/\tau_r - Nb_i)}\prod_{j=2,j\neq i}^{N}\frac{b_j}{b_j-b_i}. \tag{28}$$

The first term can be approximated with

$$\lim_{N\to\infty}\left\{\prod_{j=2}^{N}\frac{1}{1-x/(Nb_j)}\right\} = \lim_{N\to\infty}\exp\left(-\sum_{j=2}^{N}\ln\left(1-\frac{x}{Nb_j}\right)\right) = \mathrm{e}^{x\bar{\tau}} + O(1/N). \tag{29}$$

The second term can be written in terms of partial fractions to give

$$\begin{aligned}\tilde{f}_N(s) = {} & \frac{\mathrm{e}^{\bar{\tau}/\tau_r}}{1+\tau_r s} + \frac{1}{1+\tau_r s}\sum_{i=2}^{N}\left(\frac{b_i}{1/(N\tau_r)-b_i} - \frac{b_i}{-s/N-b_i}\right) \\ & \times \prod_{j=2,j\neq i}^{N}\frac{b_j}{b_j-b_i} + O(1/N).\end{aligned} \tag{30}$$

Next consider the identity

$$\lim_{N\to\infty}\left\{\sum_{i=2}^{N}\frac{b_i}{y/N-b_i}\prod_{j=1,j\neq i}^{N}\frac{b_j}{b_j-b_i}\right\} = -\mathrm{e}^{-y\bar{\tau}} + O(1/N), \tag{31}$$

which we prove in the appendix. Substituting into equation (30) and cancelling terms yields

$$\tilde{f}_N(s) = \frac{\mathrm{e}^{-s\bar{\tau}}}{1+\tau_r s} + O(1/N). \tag{32}$$

This is easily inverted using the Bromwich integral to give

$$f_N(z) \approx \begin{cases} 0 & \text{for } 0 < z < \bar{\tau}, \\ \frac{1}{\tau_r}\mathrm{e}^{(\bar{\tau}-z)/\tau_r} & \text{for } z > \bar{\tau}. \end{cases} \tag{33}$$

Note that this distribution is now only a function of τ_r and $\bar{\tau}$ suggesting that there is a simplified model with the same distribution function.

6. Distinguishable and equivalent reaction mechanisms

The distribution function (33) can now be compared with distributions of simplified reaction mechanisms. When $\tau_r \gg \bar{\tau}$ the rate limiting step is much greater than the sum of all other steps, so

$$f_N(z) \approx \frac{e^{-z/\tau_r}}{\tau_r}. \tag{34}$$

This equation is the same as the Van Slyke–Cullen model of enzyme action with one intermediate.

Next, consider the opposite case where $\tau_r \ll \bar{\tau}$. In this case, although the 'rate limiting' step is greater than any of the other single steps, the combined time of all the other steps is much greater. In this limit the distribution function becomes

$$f_N(z) \approx \delta(z - \bar{\tau}), \tag{35}$$

where $\delta(z)$ is the Dirac delta function. The rate of change of P equation (8) is then given by

$$\frac{dP}{dt} = R(t - \bar{\tau}), \tag{36}$$

which is a simple differential delay equation. This reaction can be represented schematically by

$$S + E \xrightarrow{\text{delay}} P + E, \tag{37}$$

which is a version of the Brown model for enzyme action proposed by Rousel [17].

The final case is when τ_r and $\bar{\tau}$ are approximately the same size. Consider the reaction

$$S + E \rightarrow C \xrightarrow{\text{delay}} P + E, \tag{38}$$

which is the Brown model of enzyme action [2]. The equations for the rate of change of complex and product are

$$\begin{aligned} \frac{dC}{dt} &= R(t) - \tfrac{C(t)}{\tau_r}, \\ \frac{dP}{dt} &= \tfrac{C(t-\bar{\tau})}{\tau_r}, \end{aligned} \tag{39}$$

which is a differential delay system. A simple calculation reveals that this system is equivalent to a distributed delay system with the same distribution function as equation (33).

Our analysis has demonstrated how simplified models for enzyme kinetics can be derived as limiting cases of an enzyme–substrate reaction with N-intermediates. It has also shown that the rate of product formation for reaction

mechanisms with one, two or multiple reaction mechanisms can be written in the form of a distributed delay differential equation. The shape of the distribution of the delay is determined by the relative sizes of the rates of the individual reactions, and could be employed to distinguish between tentative reaction mechanisms.

7. Discussion

The central dogma of chemical kinetics is that reaction mechanisms can never be proven, but can only be disproved. This is certainly true for enzyme catalysed reactions with multiple intermediates studied by quasi-steady-state kinetics [4, 12]. The mechanism of enzyme action is defined when all the intermediates are characterised and rate constants are determined.

Biochemists tried to detect the intermediates and to measure the rate constants with pre-steady-state kinetics experiments. If the intermediates are directly observed, their half-life and rates of decay and formation measured, experiments can be use to test a particular mechanism. One of the limitations of this approach is that on certain occasions intermediates can remain undetected due to their low concentration or they are beyond the timescale of the measurements [21].

In this paper, we wrote the rate equation for product formation in the form of a distributed delay equation for enzyme–substrate reaction mechanisms involving multiple complexes. We found that the form of distribution of the delay is characteristic for each reaction mechanism and determined the conditions under which a number of reaction mechanisms are equivalent. We must emphasise that a particular distribution of delays does not give a unique reaction mechanism, but can tell us about the number of intermediates involved in the reaction mechanism and the relative sizes of the individual rate constants. From these results, a new experimental approach could be envisaged where the distribution of the delay in product formation is studied to distinguish a particular reaction mechanism.

The imposition of delays in the governing equations of a reaction does not alter the reaction mechanism, as have been shown in a number of studies on the effects of delayed feedback in chemical systems [17, 20, 22, 26, 28]. The use of a delay feedback has previously been employed as a means by which to probe a reaction mechanism by determining the elements of the stationary-state Jacobian matrix elements and inducing oscillations by delayed feedback [29]. One of the major limitations of the latter approach is that it requires the precise measurement of chemical species which may be quite difficult to detect, such as intermediates and radicals. The new methodology we are proposing does not require the detection of intermediates: it requires the measurement of the delay resulting from the formation and decay of reaction intermediates. This paper is the first contribution to a number of new kinetic approaches leading toward the determination of reaction mechanisms for enzyme catalysed reactions.

Acknowledgments

We are very grateful to Professor P.K. Maini (Centre for Mathematical Biology, Oxford) for a critical revision of the manuscript. The authors acknowledge support from the Research Training Fellowship Programme in Mathematical Biology [Grants No. 069149 (RH) and 069155 (SS)] of the Wellcome Trust (London). Part of this document has been typed with the aid of peditPro provided by the courtesy of PaulComputing (http://www.paulcomputing.com).

Appendix

The identities (24) and (31) involving sums of products of numbers are now proved. Define the function

$$p_N(x) = \prod_{i=1}^{N}(x - k_i), \tag{A1}$$

where $N \geq 2$ and k_i are distinct and positive. Consider $1/p(x)$ expanded as a sum of partial fractions [30]:

$$\frac{1}{p(x)} - \sum_{i=1}^{N} \frac{1}{(x - k_i)p'(k_i)} = 0, \tag{A2}$$

where $p'(k_i)$ is the derivative of $p(x)$ evaluated at k_i, and $k_i \neq k_j$ when $i \neq j$. This is proved by considering the complex function $1/p(z)$, which is a meromorphic function containing n simple poles at $x = k_i$ with residue $1/p'(k_i)$. The LHS of equation (A2) is then a holomorphic function which is bounded and tends to 0 at large x, so by Liouville's theorem it is 0 in the whole complex plane. Next, take the limit $x \to \infty$ and equate powers of $O(1/x)$ (note $n \geq 2$)

$$\sum_{i=1}^{N} \frac{1}{p'(k_i)} = \sum_{i=1}^{N} \prod_{j=1, j\neq i}^{N} \frac{1}{k_i - k_j} = 0. \tag{A3}$$

Multiplying by $\prod_{i=1}^{N} k_j$ yields identity (24). Next, substitute $x = y/N$, take the limit $N \to \infty$ and equate $O(1)$ terms. The first term of equation (A2) yields

$$\prod_{i=1}^{N} \frac{1}{y/N - k_i} = \prod_{i=1}^{N} \frac{1}{-k_i} \prod_{j=1}^{N} \frac{1}{1 - y/(Nk_j)} = (-1)^N \mathrm{e}^{-y\bar{\tau}} \prod_{i=1}^{N} \frac{1}{k_i} + O(1/N), \tag{A4}$$

where

$$\bar{\tau} \equiv \frac{1}{N} \sum_{i=1}^{N} \frac{1}{k_i}, \tag{A5}$$

and we have used equation (29). Finally, insert equation (A4) into equation (A2) and re-arrange to give

$$\lim_{N\to\infty}\left\{\sum_{i=1}^{N}\frac{k_i}{y/N-k_i}\prod_{j=1,j\neq i}^{N}\frac{k_j}{k_j-k_i}\right\}=-\mathrm{e}^{-y\bar{\tau}}+O(1/N), \tag{A6}$$

which is identity (31).

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