How to Impose Microscopic Reversibility in Complex Reaction Mechanisms

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ABSTRACT Most, but not all, ion channels appear to obey the law of microscopic reversibility (or detailed balance). During the fitting of reaction mechanisms it is therefore often required that cycles in the mechanism should obey microscopic reversibility at all times. In complex reaction mechanisms, especially those that contain cubic arrangements of states, it may not be obvious how to achieve this. Three general methods for imposing microscopic reversibility are described. The first method works by setting the 'obvious' four-state cycles in the correct order. The second method, based on the idea of a spanning tree, works by finding independent cycles (which will often have more than four states) such that the order in which they are set does not matter. The third method uses linear algebra to solve for constrained rates.

INTRODUCTION

The principle of microscopic reversibility, or detailed balance, was proposed by Tolman in 1924 (see Tolman, 1938) as an adjunct to the laws of thermodynamics. It was discussed in detail by Onsager (1931) who noted that in a cyclic reaction the requirements of the first and second laws would be fulfilled if the dynamic state of equilibrium is such that that there is an overall but uniform movement around the cycle. ''However,'' says Onsager, ''the chemists are accustomed to impose a very interesting additional restriction, namely, when the equilibrium is reached each individual reaction must balance itself. They require that the transition A → B must take place just as frequently as the reverse transition B → A, etc.'' This procedure is known to chemists as 'detailed balance'. A more recent discussion is given by Denbigh (1951), and see also Kelly (1979).

The principle states that when a system is at equilibrium, the frequency of transitions is the same in both directions for each individual reaction step (see Eq. A1.5). A consequence of this is that for any cyclic reaction the product of the rate constants going one way around the cycle is equal to the product going the other way around. An example of calculation of the frequency of transitions at the single molecule level is given by Colquhoun and Hawkes (1995).

This principle is expected to be true only at genuine equilibrium (a state of zero entropy production). A steady state can be achieved that is not a genuine equilibrium (a state of minimum, but not zero, entropy production), though to maintain this steady state, some sort of external energy supply is needed (for example, an ionic gradient that is itself maintained by energy-requiring pumps).

Most of the theoretical results that have been obtained for the distributions of things like open time duration, or burst length for an ion channel (for example, those in Colquhoun and Hawkes, 1982) assume (at most) only a steady state (i.e., that nothing varies with time so for example $d\boldsymbol{p}/dt = 0$, where $\boldsymbol{p}$ is the vector of state occupancies). They make no assumption that there is a genuine equilibrium, so they are valid for irreversible reactions and do not require any assumption that microscopic reversibility holds.

In the examples given here, the smallest cycles have four states. Occasionally mechanisms with three-state cycles are postulated, though such cases usually seem to arise only when two states are treated as one. Four-state cycles have a natural physical origin. If it takes two reaction steps to get from state 1 to state 3, it is usually expected that it will take two steps (possibly not in the same order) to get back from state 3 to state 1. Nevertheless, it is possible that, for example, three conformations could exist such that a direct transition from 3 to 1 could occur. In any case, the methods given here will work for cycles of any size.

TESTS AND EXPERIMENTAL EVIDENCE

Single channel measurements can provide a much more direct test of microscopic reversibility than any other method. When microscopic reversibility holds, the channel record will be symmetrical in time—you will not be able to tell whether the tape is being played forward or backward. If microscopic reversibility is not obeyed, asymmetry might appear in the kinetics, or, more obviously, it may appear in transitions between conductance levels. Rothberg and Magleby (2001) have described three methods of looking at kinetic reversibility. For example, conditional distributions of open times that are adjacent to specified shut time (ranges) should be the same whether preceding or following shut times are used if microscopic reversibility is obeyed.

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Another approach is to fit a mechanism with and without the constraint to obey microscopic reversibility, and then use a likelihood ratio test to judge whether the latter fit is better. Methods such as these failed to show any deviation from microscopic reversibility in large conductance Ca-activated K^+ channels (Song and Magleby, 1994). The same is true for most other channels: no breach of microscopic reversibility was found in the muscle type nicotinic receptor (Colquhoun and Sakmann, 1985), or in three sorts of recombinant *N*-methyl-D-aspartate (NMDA) receptor, NR1 subunit with NR2A, NR2B, or NR2C (Stern et al., 1992), or similar native NMDA channels (Gibb and Colquhoun, 1992). On the other hand, time asymmetry in conductance transitions was detected by Richard and Miller (1990) in a 'double-barreled' chloride channel, and for one sort of NMDA receptor, NR1-NR2D (Wyllie et al., 1996). In the latter, transitions from the 35-pS level to the 17-pS level are more common than transitions from 17 to 35 pS. A similar asymmetry was found in a mutant NMDA channel (Schneggenburger and Ascher, 1997). Thus, although most channels appear to behave in a manner consistent with microscopic reversibility, it is not universal. That is hardly surprising because any interaction between ion flow (which is not at equilibrium) and gating could cause such an effect (Lauger, 1983; Finkelstein and Peskin, 1984). That seems to be what is happening in both chloride and NMDA channels.

SETTING MICROSCOPIC REVERSIBILITY

In a simple cyclic mechanism such as that shown in Fig. 1, it is easy to ensure that the rate constants obey microscopic reversibility. The fact that the product of the rates going clockwise is the same as the product going anticlockwise,

$$k_{12}k_{23}k_{34}k_{41} = k_{21}k_{14}k_{43}k_{32}, \tag{1}$$

allows one of the rate constants to be calculated from the other seven. Thus, if it is chosen to set k_{12} by microscopic reversibility then

$$k_{12} = \frac{k_{21}k_{14}k_{43}k_{32}}{k_{23}k_{34}k_{41}}. \tag{2}$$

When rate constants are being fitted to data, the seven rate constants on the right-hand side are free parameters, to be estimated, and at each iteration the corresponding value for the eighth rate, k_{12} in this example, is calculated from Eq. 2. This is what is done in fitting programs such as HJFCIT (see Colquhoun et al., 2003). The ordering method and the spanning tree method described in this article are now implemented in HJCFIT and the theory programs available from http://www.dcsite.org.uk.

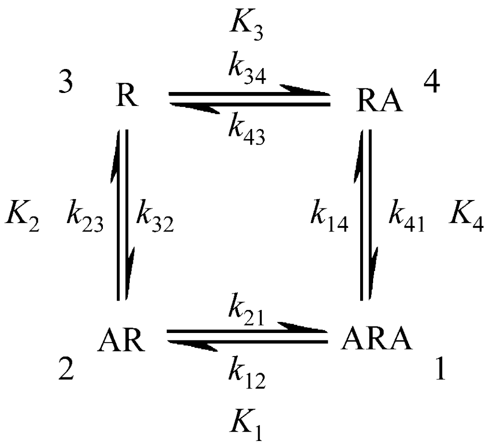

FIGURE 1 A four-state cyclic reaction mechanism. The names of the states in this example are intended to indicate that R is a receptor with two different binding sites; either (states 2 and 4) may become occupied before both are occupied (state 1). The labels on the arrows indicate the transition rates and the corresponding equilibrium constants are shown as $K_1 = k_{12}/k_{21}$, $K_2 = k_{23}/k_{32}$, $K_3 = k_{43}/k_{34}$, $K_4 = k_{14}/k_{41}$.

If we are concerned only with equilibrium constants (say $K_1 = k_{12}/k_{21}$, $K_2 = k_{23}/k_{32}$, $K_3 = k_{43}/k_{34}$, $K_4 = k_{14}/k_{41}$), then Eq. 1 implies that one of the four equilibrium constants can be calculated from the other three, thus

$$K_1 = \frac{K_3K_4}{K_2}. \tag{3}$$

In more complicated reaction schemes it may not be obvious how many rate constants are 'free' and how many are fixed by microscopic reversibility, and still less obvious how these rates should be calculated. In particular, several proposed mechanisms for the NMDA receptor, and a few that have been considered for ACh and glycine receptors, contain cubic structures, and these schemes necessitated a more systematic approach. Cubic mechanisms are commonly postulated also for receptors that are coupled to G-proteins (Weiss et al., 1996; see also Colquhoun, 1998).

There are three different methods that allow microscopic reversibility to be set in mechanisms of any complexity. The first method is to use the 'obvious' four-membered cycles as above; in this case the resulting equations are not mutually independent and the order in which cycles are set is crucial. The second method is to discover cycles that are mutually independent, so the order in which they are set does not matter (not all of them will be four-membered cycles in this case). Both methods will be described. The former method is easier to understand at an intuitive level, and easier to apply 'manually' in simple cases, but the latter is more general and also easier to program. The third method, which is described in Appendix 2, is based on solving the equations for the constraints and may be useful in cases of complicated mechanisms when many physical constraints are imposed, as well as microscopic reversibility.

SETTING MICROSCOPIC REVERSIBILITY BY ORDERING CYCLES

Consider the reaction scheme shown in Fig. 2. It represents a two-dimensional reaction scheme with 16 states (one at each vertex) and contains nine four-membered cycles (numbered 1–9 in the diagram). It is obvious that if cycles 2, 3, 4, and 5 were set first, then every connection in cycle 1 would have already been set and it would be impossible to change any of them. However, if cycle 1 is set first, then 2, 3, 4, and 5, and lastly the 'corner' cycles, 6, 7, 8, and 9, then no problems arise. The procedure is to set the cycles in order of decreasing number of shared edges. The central cycle (1) has four shared edges and so is set first. Cycles 2, 3, 4, and 5 have three shared edges and are set next (in any order), and the 'corner' cycles, 6, 7, 8, and 9, have two shared edges and are set last. This method allows microscopic reversibility to be set in all nine cycles, so nine rates are so set. There are 16 states, 24 connections (equilibrium constants), and therefore 48 rate constants, of which $48 - 9 = 39$ rates are free parameters. Note that $24 - 16 + 1 = 9$ (it is shown below, Eq. 9, that this calculation is quite generally valid).

The scheme in Fig. 2 contains many cycles with more than four members. The perimeter, for example, forms a cycle containing 12 states. It is easy to show that if the nine smallest cycles obey microscopic reversibility then any larger outer cycle that encloses them also does so. The larger cycles would usually be considered redundant. In more complicated cases it may not be obvious which cycles are 'redundant', but the spanning tree method (below) provides a simple solution to that problem.

Consider next a reaction mechanism with eight states (vertices) in a cubic arrangement, as shown in Fig. 3. This has six four-state cycles (six faces), 12 connections (edges), and 24 rate constants. If we again restrict ourselves to four-state cycles, we again find that the order in which they are set matters.

Suppose we set microscopic reversibility (as in Eq. 2) for the top, bottom, front, and back faces (cycles). This leaves the left and right faces to be set. But every rate constant in both of these faces is part of a cycle that has already been set, and which therefore cannot be changed, so only four faces can be set. If, however, we start by setting top, bottom, back, and left faces, we are left with the front and right faces, and the front face can be set because it has a link (2–3) that is not part of a cycle that has already been set. We still can't set the sixth face, but we don't need to do so because it is easily proved that if five faces obey microscopic reversibility, the sixth must also do so. Order matters: the last two faces must be adjacent, not opposite.

This sort of argument can be extended to more complex schemes. For example in a 3 × 3 × 3 stack of cubes (27 cubes, 64 states, 108 four-membered cycles, 144 connections, 288 rate constants) it is obvious that the central cube (which shares all of its faces with outer cubes) must be set first.

The need for setting cycles in a particular order arises from the fact that the equations for calculating rates from them are not independent. For example, the top face in Fig. 3 is a four-state cycle containing states 1–2–7–6. If, say, we choose to set k_{12} from this cycle we have

$$k_{12} = k_{21}\frac{k_{16}k_{67}k_{72}}{k_{27}k_{76}k_{61}}. \tag{4}$$

If we then calculate, say k_{23} from the front face (the four-state cycle 1–2–3–4) we use

$$k_{23} = k_{32}\frac{k_{21}k_{14}k_{43}}{k_{34}k_{41}k_{12}}. \tag{5}$$

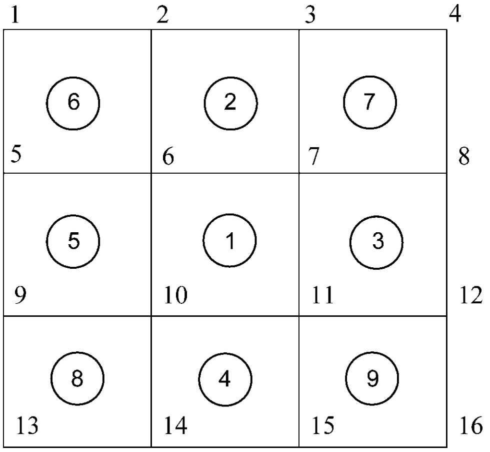

FIGURE 2 Representation of a two-dimensional reaction scheme with 16 states (one at each vertex). It contains nine four-membered cycles (numbered 1–9 in the diagram).

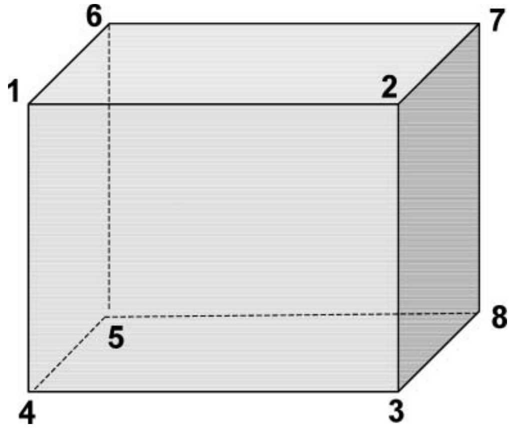

FIGURE 3 A reaction mechanism with eight states (vertices) in a cubic arrangement.

Notice that k_{12} is on the left-hand side of Eq. 4, but also occurs on the right-hand side of Eq. 5. This causes no problems as long as k_{12} is evaluated before it is needed, but it is in this sense that the equations are not independent, and that is why the order in which the cycles are set matters.

In summary, the following rules of ordering work for the setting of four-membered cycles in all cases we have encountered.

1. If the mechanism contains cycles that are not part of a cube, then these are set first, after ordering the cycles in decreasing order of the number of shared connections (edges) they have. Those with the most shared edges are set first.
2. If the mechanism contains cubes then locate them and rank them in order of increasing number of external faces.
3. Set microscopic reversibility in each cube (as above), starting with the cube with fewest external faces.
4. For each cube, before setting microscopic reversibility, order the six faces so: a), internal faces are set before external, and b), if necessary, reorder the last two of the six faces so that the last two faces are adjacent to one another, not opposite. For example, if the last two faces were (5) top and (6) bottom, then setting the top face earlier will prevent the last two faces from being opposite.

The ordering method has been illustrated only for four-state cycles. Clearly it would work equally well for three-state cycles, though for schemes that contained cycles of mixed sizes some modification might be needed. For example, a three-state cycle with three shared edges would be set before a five-state cycle with four shared edges, to make sure each cycle has at least one edge that hasn't already been set by something else.

SETTING MICROSCOPIC REVERSIBILITY BY THE SPANNING TREE METHOD

The necessity for setting cycles in a particular order arises from the fact that, when cycles always have four members, the equations for microscopic reversibility are not independent of each other (see Eqs. 4 and 5). In a quite different context, a method for choosing linearly independent cycles is well known. The answer lies in graph theory (see, for example, Gibbon, 1985), a topic that is also relevant to the existence of correlations in reaction mechanisms (Colquhoun and Hawkes, 1987).

The reaction mechanism is described as a graph, each state being a vertex and each connection between states (the pair of rate constants) being an edge. The mechanism is called a connected graph, because any state is accessible from any other, directly or indirectly. The essential idea for our purpose is the spanning tree. A tree is a connected graph that contains no cycles, and it is called a spanning tree if it connects all the states (vertices). The idea can be illustrated simply by the reaction schemes in Figs. 2 and 3.

Fig. 4 shows, as heavy lines, two examples of the many possible spanning trees for the 16-state mechanism in Fig. 2. It is well known that the number of connections (edges) in a spanning tree must always be one fewer than the number of states in the mechanism: a proof can be found in Gibbon (1985). Denoting the number of connections in the spanning tree as c_{tree} and the number of states as s,

$$c_{\text{tree}} = s - 1. \tag{6}$$

Thus, all trees in this case have 15 connections, leaving $24 - 15 = 9$ connections not in the tree. These nine

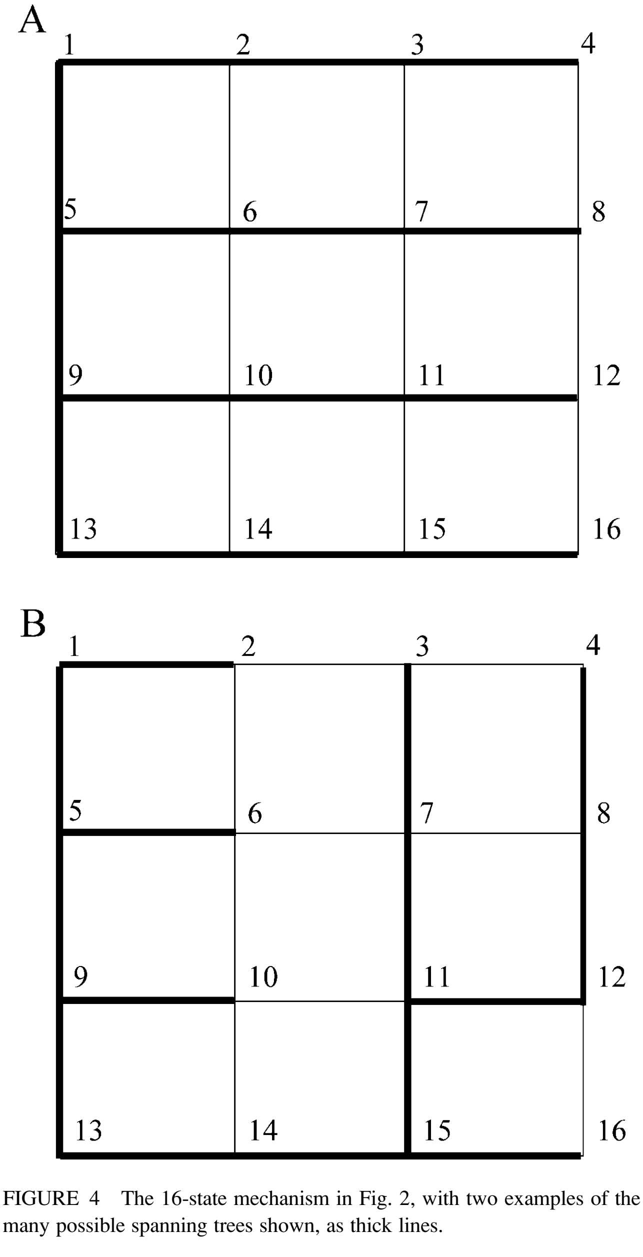

FIGURE 4 The 16-state mechanism in Fig. 2, with two examples of the many possible spanning trees shown, as thick lines.

connections are the ones that can be set by microscopic reversibility, and the cycle that is used to set them can be found by locating the (unique) path along the spanning tree that joins the two states in question. For example in Fig. 4 *A*, the connection between state 2 and 6 would be set by microscopic reversibility, and the route between them along the tree is 2–1–5–6, so if k_{26} were to be set it would be calculated as

$$k_{26} = k_{62}\frac{k_{21}k_{15}k_{56}}{k_{65}k_{51}k_{12}}. \tag{7}$$

In this case the cycle is four membered, as in Eq. 1. But to set microscopic reversibility for the connection 4–8, the route along the tree from state 4 to state 8 involves eight states (4–3–2–1–5–6–7–8), so to set, say, k_{48} we would use

$$k_{48} = k_{84}\frac{k_{43}k_{32}k_{21}k_{15}k_{56}k_{67}k_{78}}{k_{87}k_{76}k_{65}k_{51}k_{12}k_{23}k_{34}}. \tag{8}$$

In this example there are three cycles with four states, three cycles with six states, and three cycles with eight states. Fig. 4 *B* shows another possible spanning tree. This one gives rise to five four-state cycles, two six-state cycles, one eight-state cycle, and one 10-state cycle. The 10-state cycle arises when microscopic reversibility is set for the 2–3 route: the route from state 2 to state 3 is seen to be 2–1–5–9–13–14–15–11–7–3.

If all nine expressions of the sort above are written out, it is seen that none of the nine rate constants that are being calculated (on the left-hand sides) appears on the right-hand sides of the equations. In this sense the equations are independent, and the order in which they are set is irrelevant.

The proof that this method can be applied to find independent cycles in any reaction mechanism can be found in Gibbon (1985), who gives:

> Theorem 2.7 ''A set of fundamental circuits, with respect to some spanning tree of a graph *G*, forms a basis for the circuit space of *G*.''
>
> Corollary 2.1 ''The circuit space for a graph with *e* edges and *v* vertices has dimension ($e - v + 1$).''

Translated into our language, these mean that, quite generally: 1), the cycles found from the spanning tree are independent, so the order in which they are calculated does not matter, and 2), the number of connections that are set by microscopic reversibility, $c_{\rm mr}$, for any mechanism is

$$c_{\rm mr} = c_{\rm tot} - c_{\rm tree} = c_{\rm tot} - s + 1, \tag{9}$$

where $c_{\rm tot}$ is the total number of connections in the reaction mechanism.

This shows that microscopic reversibility can be set, regardless of order, in the independent cycles identified by the spanning tree. But can we be sure that doing this will ensure that all of the other cycles that can be found (often very numerous) will also obey microscopic reversibility? A formal demonstration that this is true is given in Appendix 1, which extends theorem 2.7 in Gibbon (1985) to show that if microscopic reversibility is obeyed in the fundamental cycles then it will also be obeyed for any cycle found by combining them. A stochastic argument that leads to the same conclusion is also given in Appendix 1.

In the examples in Fig. 4, the number of connections that are set by microscopic reversibility is thus $24 - 16 + 1 = 9$, as already found by the ordering argument.

The cubic mechanism (Fig. 3) has $s = 8$ states, so from Eq. 6 all spanning trees must have $c_{\rm tree} = 7$ connections, and from Eq. 9 the number of connections set by microscopic reversibility is $12 - 8 + 1 = 5$, as found by a different argument in the first section. Fig. 5 shows the cubic mechanism in Fig. 3, projected in two dimensions, and the thick lines show one of the many possible spanning trees. For this particular case, the five connections to be set by microscopic reversibility are 5–6, 7–8, 2–7, 7–8, and 3–8. The cycles for setting these are all four-state cycles apart from 7–8, for which the cycle is six-state, 7–6–1–4–5–8.

PRACTICAL IMPLEMENTATION AND THE INCORPORATION OF PHYSICAL CONSTRAINTS

In practice one often wishes to constrain the values of some of the rate constants, for physical reasons. For example, some rate constants may be set to be equal to ensure that binding of a ligand to one binding site is independent of binding to another site. It may also be required to set a particular rate constant to produce a specified EC_{50} (examples are given by Colquhoun et al., 2003; Hatton et al., 2003). A rate constant cannot be set by such a physical constraint if it is set by microscopic reversibility, so we may wish to construct a particular spanning tree, one that contains

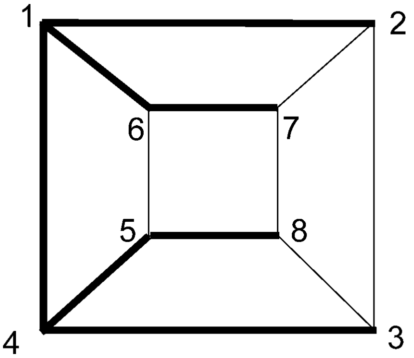

FIGURE 5 The cubic mechanism in Fig. 3 is shown projected in two dimensions, with thick lines indicating one of the many possible spanning trees.

specified connections, namely all those connections that one wishes to constrain.

There is a beautifully simple way of doing this that is easy to implement in a computer program. In our problem the connections between states are logical rather than physical, but in many applications the connections (edges) have a number such as a length or a cost attached to them. For example, a common application is to design a road or railway network to connect several towns. In such cases the spanning tree with minimum length, or minimum cost, is required. This is known as a 'minimum spanning tree'. Very efficient algorithms exist for finding minimum spanning trees (Gibbon, 1985); Prim's algorithm (Prim, 1957) is a well-known one. To force particular connections to be part of the spanning tree, so that we may constrain them later, we assign to them an imaginary length that is shorter than the length for the connections that are to be set by microscopic reversibility, and then use Prim's algorithm. If a tree exists that contains all the required routes, this method will find it.

Similarly it is possible to specify which connections you would like to set by microscopic reversibility by assigning them a longer imaginary length so that a spanning tree will be found that excludes the specified connections if that is possible. For example, the following values will guarantee a spanning tree with the right properties if such a tree exists: 'length' $= 1$ for routes that are to be included in the tree, 'length' $= 2$ for other routes apart from 'length' $= s$ (the number of states) for routes that one wishes to set by microscopic reversibility.

As an example of constraints, consider the simple case of binding to two different binding sites, which can be represented as shown in Fig. 1. In the case where the sites are independent, so binding to one is unaffected by whether or not the other is occupied, we wish to apply the constraints

$$k_{14} = k_{23} \tag{10}$$

$$k_{41} = k_{32} \tag{11}$$

$$k_{12} = k_{43} \tag{12}$$

$$k_{21} = k_{34}. \tag{13}$$

In Colquhoun et al. (2003), k_{14}, k_{41}, and k_{12} were found from the constraints (Eqs. 10–12), and k_{21} was then set by microscopic reversibility (a procedure that ensures the fourth constraint, Eq. 13, is also obeyed). In more complex mechanisms it may not be at all obvious which rates to constrain and which to set by microscopic reversibility. In cases like this, in which the constraints alone are sufficient to ensure microscopic reversibility, it is much simpler to set all the constraints required by the physical problem (four in this example), and to ignore spanning trees altogether. In difficult cases it may be necessary to use the general method described in Appendix 2.

APPENDIX 1: PROOF THAT SETTING THE FUNDAMENTAL CYCLES ENSURES THAT ALL CYCLES OBEY MICROSCOPIC REVERSIBILITY

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We need to prove that once microscopic reversibility has been set in the fundamental cycles that are identified by the spanning tree, then all other possible cycles will also obey microscopic reversibility, and so need not be considered. We shall give two proofs, because each casts light on the problem from a different point of view. The first proof is based purely on graph theory, and the second takes a more stochastic approach.

Proof that combination of fundamental cycles preserves microscopic reversibility

Theorem 2.7 in Gibbon (1985) tells us that we can obtain any circuit as the 'sum' of fundamental circuits, where 'sum' is defined as addition of the edges modulo 2 and is usually denoted by the operator $\oplus$. Thus, if C_1 and C_2 are circuits $C_1 \oplus C_2$ is a circuit or edge disjoint union of circuits formed by those edges in C_1 or C_2, but not both. To use this result to set microscopic reversibility via the spanning tree method we also need to show that $\oplus$ preserves the microscopic reversibility property, i.e., we need to show that if C_1 and C_2 satisfy the necessary conditions then so does $C_1 \oplus C_2$.

To prove this we will make use of the following definitions and notation. Suppose we a have a circuit C_i. We define a 'consistent orientation' of C_i to be an allocation of a direction to each of the edges of C_i such that we can traverse the complete circuit following the edges in a forward direction. Any circuit will have two consistent orientations, one of which will be the reverse of the other. (We can obviously extend the definition to the case where C_i is an edge disjoint union of circuits.)

For a given consistent orientation let $d_{\mathrm{i}}^{+}(e)$ be the rate constant associated with edge e in the forward direction for that orientation, and $d_{\mathrm{i}}^{-}(e)$ be the rate constant for edge e in the opposite direction.

Consider $C_3 = C_1 \oplus C_2$. We will start by showing that if we orient the edges in C_3 according to a consistent orientation, then for consistent orientations of C_1 and C_2 the remaining edges must have opposite orientations in the two circuits. We then show that this implies that if C_1 and C_2 satisfy microscopic reversibility so does C_3.

The edges in C_1 and C_2 can be partitioned into three classes, $E_1 =$ those edges in C_1 but not C_2, $E_2 =$ those edges in C_2 but not C_1, and $E_{\mathrm{B}} =$ those edges in both. Let G be the graph given by $E_1 \cup E_2 \cup E_{\mathrm{B}}$. This will consist of $C_3 = E_1 \cup E_2$ (by definition) and the set of edges in E_{B}. As C_1 and C_2 are circuits E_{B} must be a path or disjoint union of paths each starting and ending at a vertex of C_3. Consider any such path joining v_i and v_j. v_i will be a vertex of degree 3 in G and will represent a point in C_3 where C_1 and C_2 meet.

Now assume that we direct the edges in E_1 and E_2 so that we have a consistent orientation of C_3. Either the edge from C_1 or the edge from C_2 will be oriented into v_i and the other will be oriented away from v_i. Assume without loss of generality that it is the edge from C_1 that is oriented toward v_i. Then in a consistent orientation of C_1 the edges of the path in E_{B} must be oriented from v_i to v_j. Similarly as the edge from C_2 is oriented away from v_i, in a consistent orientation of C_2 the edges in the path must be oriented from v_j to v_i. Thus the edges in the path will be oriented in opposite directions in C_1 and C_2, and this will be true of all such paths.

Thus if we orient the edges in E_1 and E_2 so that they form a consistent orientation in C_3 the edges in E_{B} will be oriented in opposite directions in the corresponding consistent orientations of C_1 and C_2 and the following equalities apply.

$$d_1^{+}(e) = d_3^{+}(e) \quad \text{and} \quad d_1^{-}(e) = d_3^{-}(e) \forall e \in E_1 \tag{A1.1}$$

$$d_2^{+}(e) = d_3^{+}(e) \quad \text{and} \quad d_2^{-}(e) = d_3^{-}(e) \forall e \in E_2 \tag{A1.2}$$

$$d_1^+(e) = d_2^-(e) \quad \text{and} \quad d_1^-(e) = d_2^+(e) \forall e \in E_B. \qquad \text{(A1.3)}$$

Now assume C_1 and C_2 satisfy the conditions for microscopic reversibility. We have:

$$\prod_{e \in E_1} d_1^+(e) \cdot \prod_{e \in E_B} d_1^+(e) = \prod_{e \in E_1} d_1^-(e) \cdot \prod_{e \in E_B} d_1^-(e) \quad \text{and} \quad \prod_{e \in E_2} d_2^+(e) \cdot \prod_{e \in E_B} d_2^+(e) = \prod_{e \in E_2} d_2^-(e) \cdot \prod_{e \in E_B} d_2^-(e)$$
$$\Rightarrow \prod_{e \in E_1} d_1^+(e) \cdot \prod_{e \in E_2} d_2^+(e) \cdot \prod_{e \in E_B} d_1^+(e) \cdot \prod_{e \in E_B} d_2^+(e) = \prod_{e \in E_1} d_1^-(e) \cdot \prod_{e \in E_2} d_2^-(e) \cdot \prod_{e \in E_B} d_1^-(e) \cdot \prod_{e \in E_B} d_2^-(e)$$

Substituting from Eqs. A1.1, A1.2, and A1.3 gives:

$$\prod_{e \in E_1} d_3^+(e) \cdot \prod_{e \in E_2} d_3^+(e) \cdot \prod_{e \in E_B} d_2^-(e) \cdot \prod_{e \in E_B} d_2^+(e) = \prod_{e \in E_1} d_3^-(e) \cdot \prod_{e \in E_2} d_3^-(e) \cdot \prod_{e \in E_B} d_2^+(e) \cdot \prod_{e \in E_B} d_2^-(e)$$
$$\Rightarrow \prod_{e \in E_1} d_3^+(e) \cdot \prod_{e \in E_2} d_3^+(e) = \prod_{e \in E_1} d_3^-(e) \cdot \prod_{e \in E_2} d_3^-(e) \Rightarrow \prod_{e \in C_3} d_3^+(e) = \prod_{e \in C_3} d_3^-(e). \qquad \text{(A1.4)}$$

Equation A1.4 shows that the microscopic reversibility condition is indeed satisfied by C_3, as required.

By repeatedly applying the above result we have the required property that any sum of a set of circuits satisfying microscopic reversibility will also satisfy the condition.

A stochastic proof that setting the fundamental cycles ensures microscopic reversibility

Kelly (1979) states, in his theorem 1.3: ''A stationary Markov process is reversible if and only if there exists a collection of positive numbers $p_j, j \in S$, summing to unity that satisfy the detailed balance conditions

$$p_j q_{jk} = p_k q_{kj}, \quad j, k \in S. \qquad \text{(A1.5)}$$

When there exists such a collection $p_j, j \in S$, it is the equilibrium distribution of the process.'' Here, S denotes the set of all states. In our language, p_j represents the fractional occupancy of state j, and q_{jk} is the transition rate from state j to state k (an element of the $\boldsymbol{Q}$ matrix), so Eq. A1.5 states that, for a reversible process at equilibrium, the frequency of transitions is the same in each direction for every individual reaction step. This implies that the products of rates going each way around a cycle must be equal, as exemplified in Eq. 1 (e.g., Kelly, 1979; theorem 1.8).

To prove that setting the fundamental cycles ensures microscopic reversibility, we find a collection $p_j, j \in S$, such that Eq. A1.5 is satisfied. First note that there exists a unique collection $p_j, j \in S$, such that Eq. A1.5 is satisfied for the links in the tree. Indeed, these p_j are the equilibrium occupancies for the process that has only the links in the tree, which is reversible by Kelly (1979), Lemma 1.5. The setting equations (exemplified in Eqs. 2, 7, and 8), with these p_j, show that Eq. A1.5 also holds for any link that is not in the spanning tree, so the process is reversible and the p_j represent the equilibrium occupancies for our reaction scheme.

We can illustrate the above proof by considering the spanning tree in Fig. 4 *B*. The p_j can be constructed explicitly as follows. Applying Eq. A1.5 to the link 1–5 yields

$$p_5 = \frac{k_{15}}{k_{51}} p_1. \qquad \text{(A1.6)}$$

Now applying it to the link 5–6 yields

$$p_6 = \frac{k_{56}}{k_{65}} p_5 = \frac{k_{56} k_{15}}{k_{65} k_{51}} p_1. \qquad \text{(A1.7)}$$

Continuing in this fashion yields each of $p_2, p_3, \ldots, p_{16}$ as a multiple of p_1, which can then be determined since the p_j sum to one. Now consider a link that is in the mechanism but not in the spanning tree, for example 2–6. The rate k_{26} is set using Eq. 7. Applying Eq. A1.5 to the links 1–2, 1–5, and 5–6, which are in the spanning tree, implies that

$$\frac{k_{21}}{k_{12}} = \frac{p_1}{p_2}, \quad \frac{k_{15}}{k_{51}} = \frac{p_5}{p_1}, \quad \text{and} \quad \frac{k_{56}}{k_{65}} = \frac{p_6}{p_5}. \qquad \text{(A1.8)}$$

Substituting these into Eq. 7 yields $p_2 k_{26} = p_6 k_{62}$, so Eq. A1.5 holds also for the link 2–6. A similar argument shows that Eq. A1.5 holds for all the other links that are not in the spanning tree. Note that the above proof indicates that for a reversible process, the equilibrium occupancies depend only on the equilibrium constants.

APPENDIX 2: CONSTRAINTS AS A LINEAR SYSTEM OF EQUATIONS

The question of how to apply physical constraints, in addition to microscopic reversibility, was discussed in the last section of the article. The example used there can be used to illustrate a general way to deal with this sort of problem.

In the example, it was found that the four physical constraints, Eqs. 10–13, implied that microscopic reversibility, Eq. 1, is satisfied. This means that these five equations are not independent. This can be shown as a general property of linear systems of equations. To do this we shall use the logarithms of the rate constants, denoted $x = \log(k)$. In the example there are eight rate constants, the unknowns that are to be estimated. The eight $\log(k)$ values can be arranged in an 8×1 column vector, $\boldsymbol{x}$. The order does not matter, but we can choose to start from k_{12} and proceed clockwise up to k_{41} and then anticlockwise from k_{21} up to k_{32}.

Thus we define

$$x_1 = \log(k_{12}), \; x_2 = \log(k_{23}), \ldots,$$
$$x_5 = \log(k_{21}), \ldots, \; x_8 = \log(k_{32}). \qquad \text{(A2.1)}$$

In this notation the constraints in Eqs. 10–13 are

$$\begin{aligned} x_6 - x_2 &= 0 \\ x_4 - x_8 &= 0 \\ x_1 - x_7 &= 0 \\ x_5 - x_3 &= 0. \end{aligned} \tag{A2.2}$$

In addition the constraint of microscopic reversibility can be written as a fifth equation,

$$x_1 + x_2 + x_3 + x_4 - x_5 - x_6 - x_7 - x_8 = 0. \tag{A2.3}$$

These constitute a homogeneous system of five linear equations with eight unknowns, which can be written as

$$\sum_{j=1}^{8} a_{ij} x_j = 0 \quad i = 1, \ldots, 5. \tag{A2.4}$$

This can be written generally in matrix form as

$$\boldsymbol{Ax} = \mathbf{0}, \tag{A2.5}$$

where the coefficients, a_{ij}, are elements of the 5×8 matrix, $\boldsymbol{A}$, namely

$$\boldsymbol{A} = \begin{bmatrix} 0 & -1 & 0 & 0 & 0 & 1 & 0 & 0 \\ 0 & 0 & 0 & 1 & 0 & 0 & 0 & -1 \\ 1 & 0 & 0 & 0 & 0 & 0 & -1 & 0 \\ 0 & 0 & -1 & 0 & 1 & 0 & 0 & 0 \\ 1 & 1 & 1 & 1 & -1 & -1 & -1 & -1 \end{bmatrix}. \tag{A2.6}$$

This matrix has rank 4, rather than the maximum possible rank of 5 for a 5×8 matrix. This is exactly what was expected, because the five equations above are not linearly independent. This means, in our context, that four variables are determined by the constraints, leaving the other four as free parameters the values of which must be given (e.g., they might be the values of the free rate constants to be tried by a fitting program).

In the case of the cubic mechanism shown in Fig. 3, there are six 4-state cycles, and 24 rate constants. The six microscopic reversibility constraints can be written as six equations analogous with Eq. A2.3. When this is done the matrix, $\boldsymbol{A}$, of coefficients is 6×24 and is found to have rank 5. Thus, as found above, five rate constants are determined by the microscopic reversibility constraints leaving 19 free rate constants. Any further constraints each add an extra row to $\boldsymbol{A}$, the rank of which will indicate the number of free parameters.

In general we will have a system with n unknowns (the total number of rate constants in the model) and m constraints (the sum of the microscopic reversibility constraints and the constraints to be imposed on some of the rate constants). The number of independent constraints will be equal to the rank r of the $m \times n$ matrix, $\boldsymbol{A}$, ($r \leq m$). The number of free parameters is therefore $n - r$. In a very complicated model with many cycles and physical constraints to be imposed, the rank of the matrix of the associated linear system may be the simplest way of determining the number of free parameters.

We wish to find a general way to compute the r constrained rates from $n - r$ free rates. The theory of homogeneous linear systems (e.g., Schneider and Barker, 1973) gives a solution as follows. Define $\boldsymbol{B}$ as any $r \times r$ submatrix of $\boldsymbol{A}$ that has a nonzero determinant, and is therefore invertible. In general there will be several such 'nonnull minors', and which one of them is chosen will dictate which rates are calculated via constraints, just as choice of one or another of the possible spanning trees does. After interchanging rows and columns of $\boldsymbol{A}$ if necessary, we can then write $\boldsymbol{A}$ in the partitioned form

$$\boldsymbol{A} = \begin{bmatrix} \boldsymbol{B} & \boldsymbol{C} \\ \boldsymbol{D} & \boldsymbol{E} \end{bmatrix}, \tag{A2.7}$$

where $\boldsymbol{C}$ is $r \times (n - r)$. The vector $\boldsymbol{x}$ is partitioned accordingly into

$$\boldsymbol{x} = \begin{bmatrix} \boldsymbol{x}_{\mathrm{c}} \\ \boldsymbol{x}_{\mathrm{f}} \end{bmatrix}, \tag{A2.8}$$

where $\boldsymbol{x}_{\mathrm{c}}$ contains the r constrained log(rate) values, and $\boldsymbol{x}_{\mathrm{f}}$ contains $n - r$ free values.

From Eq. 2.5, $\boldsymbol{Ax} = \mathbf{0}$, is equivalent to

$$\boldsymbol{Bx}_{\mathrm{c}} + \boldsymbol{Cx}_{\mathrm{f}} = \mathbf{0}, \tag{A2.9}$$

and the solution of this is

$$\boldsymbol{x}_{\mathrm{c}} = -\boldsymbol{B}^{-1}\boldsymbol{Cx}_{\mathrm{f}}. \tag{A2.10}$$

This provides a general way of calculating the constrained values from the free ones.

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