Catecholamine Synthesis: A Hierarchy of Controls?

Peter R. Dunkley and Phillip W. Dickson

School of Biomedical Sciences, University of Newcastle, NSW 2308

The catecholamines dopamine, noradrenaline and adrenaline are neurotransmitters in the nervous system. Noradrenaline and adrenaline are also hormones in the endocrine system. Overall these catecholamines control a wide range of physiological functions, including motor coordination, blood pressure and carbohydrate metabolism. The secretion of catecholamines from neurones in the brain and from adrenal chromaffin cells in the adrenal medulla is induced by depolarisation. This leads to the opening of voltage-sensitive calcium channels. A rise in the level of calcium in the immediate vicinity of docked vesicles induces their fusion with the plasma membrane and secretion of catecholamines. However, the level of catecholamines within the nerves and the adrenal gland is maintained at a constant level despite this catecholamine secretion. There must therefore be a mechanism to control catecholamine synthesis that is closely linked to secretion. The entry of calcium into the cell has been implicated as one such control mechanism.

Tyrosine hydroxylase

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the synthesis of catecholamines (**Fig. 1**). The activity of this enzyme is regulated at the levels of transcription and translation, by feedback inhibition of catecholamines and by protein phosphorylation (1). In cells, an increase in TH phosphorylation increases the rate of catecholamine synthesis. This regulatory mechanism occurs in parallel with secretion that leads to maintenance of catecholamine levels. There are four phosphorylation sites on the N-terminal regulatory domain of TH; Ser8, Ser19, Ser31 and Ser40 (2). In this article we will focus on the phosphorylation of Ser19 and Ser40. Much of the detail of these events is omitted due to space limitations.

In resting cells the level of intracellular calcium is low. The stoichiometry of phosphorylation of TH under resting conditions depends on the cells studied. For PC12 cells, a rat phaeochromocytoma cell line that secretes catecholamines, the stoichiometry is only 0.05 and 0.03 for Ser19 and Ser40, respectively (3). Most of the TH is therefore not phosphorylated. On depolarisation, calcium that enters the cell via the voltage-sensitive calcium channels, diffuses from the plasma membrane and leads to a rise in cytosolic calcium. This leads to a rapid increase in the phosphorylation of TH. Ser19 phosphorylation is increased to a maximum within 15 seconds, while Ser40 approaches maximum phosphorylation by 4 minutes (4). The stoichiometry of phosphorylation of Ser19 at its maximum is approximately four-fold higher than in resting cells, while that of Ser40 is only increased by approximately 50% (4). The mechanisms whereby the increase in cytoplasmic calcium leads to TH phosphorylation (**Fig. 2**) have been established (4,5). The rise in intracellular calcium firstly increases the binding of calcium to calmodulin and this in turn leads to activation of calcium and calmodulin-stimulated protein kinase II (CaMPKII). Ser19 on TH can be phosphorylated directly by CaMPKII. The increase in calcium-calmodulin also leads to activation of adenylyl cyclase, which leads to an increase in cyclic-AMP and protein kinase A (PKA) activity. PKA phosphorylates Ser40 on TH. Two different signalling pathways therefore lead to phosphorylation of Ser19 and Ser40.

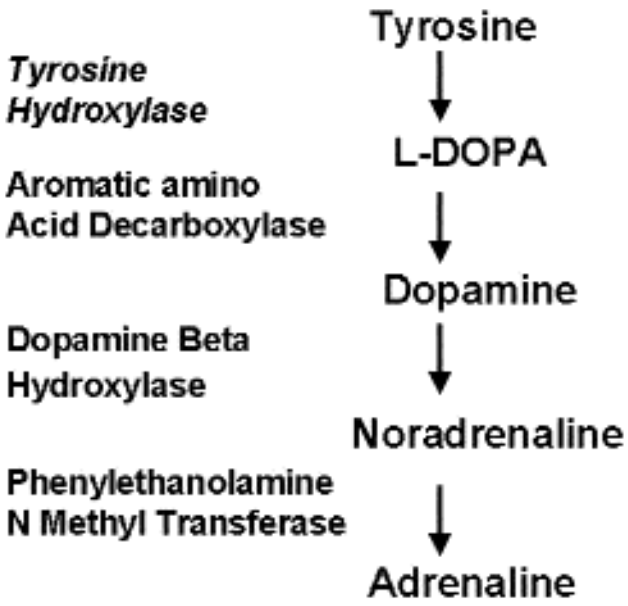

Fig. 1. Tyrosine hydroxylase is the rate-limiting enzyme in the synthesis of catecholamines.

Depolarisation of adrenal chromaffin cells increases the activity of TH up to two-fold and this increase parallels the slower phosphorylation of Ser40 and not the more rapid phosphorylation of Ser19 (5). An increase in the frequency of depolarisation further increases the entry of calcium into the cell and increases TH phosphorylation and catecholamine synthesis. A graded response of catecholamine synthesis therefore occurs depending on the level of calcium that enters the cell. The phosphorylation of Ser19 closely follows the levels of intracellular calcium. In 1992, Haycock and Wakade found that the extent of phosphorylation of Ser19 in adrenal chromaffin cells was doubled by increasing the frequency of splanchnic nerve firing from 1Hz for 5 minutes to 10 Hz for 30 seconds (6).

These findings raise a number of important questions. Why should the phosphorylation of Ser19 on TH occur so rapidly and to a greater extent than the phosphorylation of Ser40? Why is it that phosphorylation of Ser19 parallels the levels of intracellular calcium, while the phosphorylation of Ser40 parallels the increase in catecholamine synthesis?

Hierarchical phosphorylation of proteins

Covalent attachment of a phosphate group to a protein and the subsequent change in activity of the target protein is a key mechanism in the regulation of many biological pathways in all eukaryotic cells. A number of protein kinases will catalyse a very specific reaction. However, many kinases are multi-functional, that is they will phosphorylate a large number of different substrates. This phosphorylation of substrates produces a coordinated cellular response. On the other hand different signalling pathways can operate through the same multifunctional kinases. As an example, CaMPKII has been

implicated in many neuronal processes, including development, neuronal pathway formation, neurotransmitter secretion and neuronal plasticity. In each circumstance different substrates are important and yet the same signalling pathway is involved. How do the appropriate substrates get activated at the appropriate time? It is clear that the cell surface signals, the expression of the substrate and its location, as well as protein phosphatases and a range of binding proteins all play important roles. However, most phosphoproteins are phosphorylated on more than one site and it is possible that hierarchical phosphorylation of proteins is a fundamental mechanism of control in biological systems.

Hierarchical phosphorylation is defined as the phosphorylation of a protein at one site leading to an increased rate of phosphorylation at another site on the same protein (**Fig. 3**). Imagine that two signalling pathways are activated in one cell and each pathway leads to the phosphorylation of a specific one of two sites on the same substrate. If there was no hierarchical phosphorylation, then the two sites would be phosphorylated independently and the rate of formation of the doubly phosphorylated substrate would be the same no matter which site was phosphorylated first. However, if hierarchical phosphorylation existed then the presence of phosphate on one site rather than the other would matter. As seen in **Fig. 3**, if site 1 were phosphorylated then the doubly phosphorylated protein would be achieved more quickly than if site 2 were phosphorylated, as hierarchical phosphorylation occurs for site 2 and not for site 1.

An extreme example of hierarchical phosphorylation would be when the rate of phosphorylation of site 2 (reaction B in **Fig. 3**) was so slow that it would only occur when site1 was already phosphorylated (reaction C in **Fig. 3**). An example of such an extreme hierarchical phosphorylation system is the regulation of glycogen synthase. This enzyme requires the sequential phosphorylation by casein kinase II and glycogen synthase kinase (GSK-3) to inactivate the enzyme. This is achieved by the phosphorylation of glycogen synthase by casein kinase II that changes the conformation of the enzyme and forms the recognition sequence for GSK-3. Once GSK-3 has phosphorylated glycogen synthase, four more GSK-3 sites are sequentially phosphorylated and glycogen synthase is finally inactivated (7). Many of the known examples of hierarchical phosphorylation are extreme situations, which are the most simple to detect.

A more likely situation in biology would be if site 2 was phosphorylated slowly and site 1 simply increased the rate of site 2 phosphorylation (**Fig. 3**). This increase in rate might be more difficult to detect and the consequences may be subtler. If the biological outcome was dependent only on site 2 being phosphorylated and reaction B was slow, then phosphorylation of site 1 first would increase the rate of phosphorylation of site 2 and thereby increase the rate of the biological outcome being achieved. We think that this is what happens with TH.

There is increasing evidence that hierarchical phosphorylation is important in controlling biological outcomes. Cell division relies on the properly timed activation and destruction of certain regulatory proteins. Nash *et al.* have recently shown that the phosphorylation of the protein Sic1 in yeast requires the sequential phosphorylation of at least six sites before it can be ubiquitinylated and moved to the proteasome for destruction (8). This then allows the movement of the cells from G1 to S phase. The timing and sequence of the phosphorylation of all these sites acts as a molecular timer to delay this fundamental transition.

Hierarchical phosphorylation of tyrosine hydroxylase

Depolarisation of catecholaminergic cells leads to the phosphorylation of Ser19 via one signalling pathway, followed by the phosphorylation of Ser40 via another pathway. We developed a hypothesis that the role of phosphorylation of Ser19 may be to alter the conformation of TH to allow more rapid phosphorylation of Ser40. If TH was the protein shown in **Fig. 3** then Ser19 would be site 1 and Ser40 site 2. We therefore examined the effect of Ser19 phosphorylation on Ser40 phosphorylation using cloned TH *in vitro*. In initial experiments we showed that Ser19 phosphorylation induced a significant conformational change in

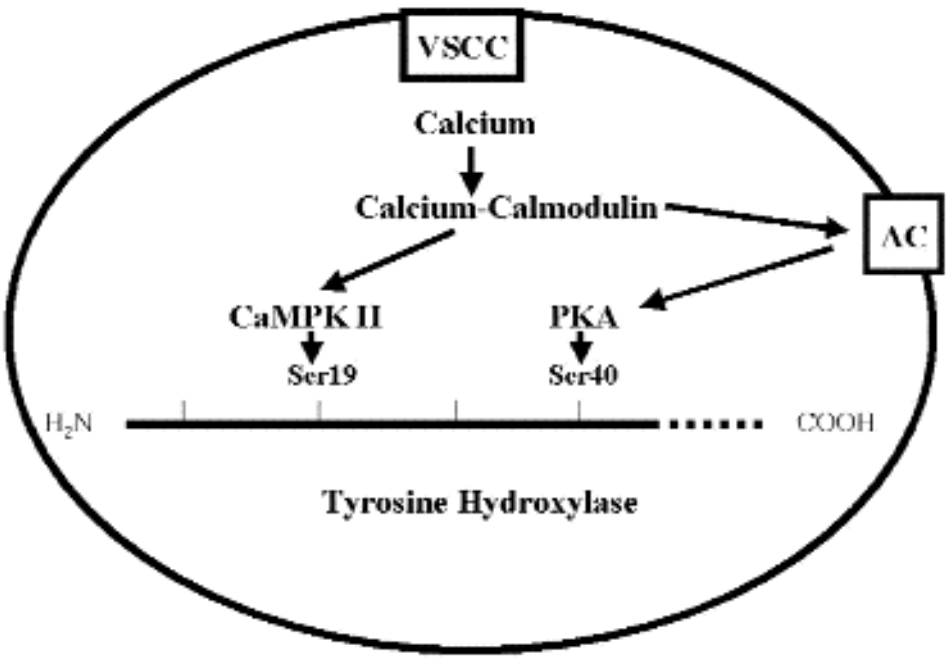

Fig. 2. The phosphorylation of tyrosine hydroxylase. Depolarisation of cells activates voltage-sensitive calcium channels (VSCC) to allow the entry of extracellular calcium. This leads to the formation of calcium-calmodulin complexes that are able to activate calcium and calmodulin stimulated protein kinase II (CaMPKII), leading to the phosphorylation of Ser19 on tyrosine hydroxylase. Adenylyl cyclase (AC) is also activated producing cyclic AMP, which activates protein kinase A (PKA) leading to the phosphorylation of Ser40 on tyrosine hydroxylase.

TH (9). Experiments to examine a direct effect of Ser19 phosphorylation on the rate of Ser40 phosphorylation were then undertaken. Over the last couple of years we have been developing methodologies that utilise mass spectrometry to investigate phosphorylation of TH (10). These methods allowed us to determine the proportion of TH molecules that had none, one or two phosphates present. We then utilised this technology, combined with the use of a mutant clone of TH in which Ser40 had been replaced with Ala, to determine the dependency of phosphorylation of one site on the phosphorylation of the other (9). We found that the rate at which Ser40 is phosphorylated if Ser19 is already phosphorylated (reaction C in **Fig. 3**) is at least three times greater than the rate at which Ser40 is phosphorylated if Ser19 is not phosphorylated (reaction B in **Fig. 3**). In addition we found that the rate of Ser19 phosphorylation (reaction A in **Fig. 3**) was not increased by prior phosphorylation of Ser40 (reaction D in **Fig. 3**). This provided the first evidence for hierarchical phosphorylation of TH. Others have since confirmed our results both qualitatively and quantitatively using a different experimental approach (11).

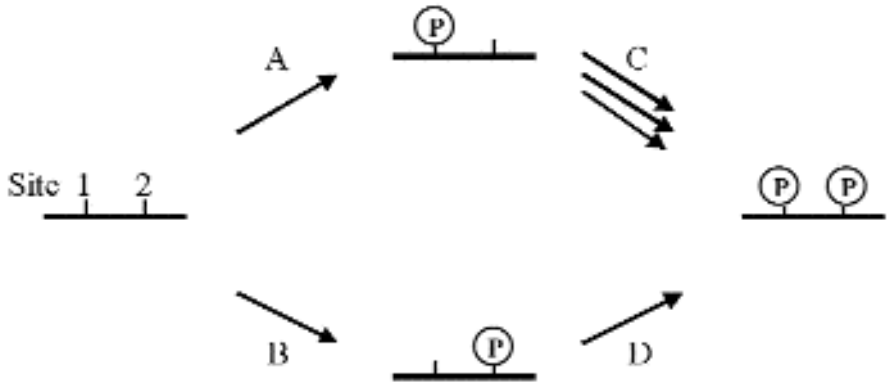

Fig. 3. Hierarchical phosphorylation of proteins.
A dephosphorylated protein can be incubated with two protein kinases that lead to phosphorylation at either site 1 or site 2 (denoted by a circled P). If both kinases are active then undergoing reaction A or reaction B will randomly phosphorylate the substrate at either site 1 or site 2 first. Further incubation will lead to the singly phosphorylated protein undergoing reaction C or D next, leading to doubly phosphorylated protein. If no hierarchical phosphorylation exists, then the rate of reaction could equal the rate of reaction B, and the rate of reaction D would equal the rate of reaction A. However, if hierarchical phosphorylation of site 2 occurs due to the prior phosphorylation of site 1, then the rate of reaction C will be increased above that of reaction B, as shown here.

Depolarisation of cells leads to two signalling pathways being activated with CaMPKII phosphorylating Ser19 and PKA phosphorylating Ser40 (**Fig. 2**). Both of these pathways are activated by calcium and calmodulin. This activation of both pathways allows for the possibility of hierarchical phosphorylation, whereas if only the PKA pathway were activated then no hierarchical phosphorylation could occur. Furthermore, Ser19 is phosphorylated faster and to a greater extent than is the case for Ser40. These conditions all favour hierarchical phosphorylation of TH.

Summary

Depolarisation of cells leads to the entry of calcium and the secretion of catecholamines. This same calcium signal also leads to an increase in the phosphorylation of TH and an increase in catecholamine synthesis. We recently found that the activity of TH isolated from adrenal chromaffin cells was increased as a result of phosphorylation of Ser19 prior to the phosphorylation of Ser40 (unpublished data). This would suggest that there is a functional significance for hierarchical phosphorylation in respect to TH activation. However, the details of this mechanism of hierarchical activation of TH still need to be determined. It has been known for over 10 years that TH was phosphorylated at multiple sites in cells. However, hierarchical phosphorylation was not detected until recently. This is because the hierarchical phosphorylation was not an extreme example where the phosphorylation of one site depended on the phosphorylation of a second site. Rather there was a three-fold increase in rate of phosphorylation of one site by the prior phosphorylation of another site, an aspect of regulation that was not detected in earlier studies.

Multiple levels of regulation act to control all biological processes. A hierarchy of established controls exists over all biological processes. To date, most emphasis has been on the role of receptors, the signalling pathways, binding proteins and protein phosphatases. It is likely however that hierarchical phosphorylation is a general biological control mechanism for proteins with multiple phosphorylation sites. The importance of hierarchical phosphorylation is that it provides the substrate with a measure of control over its own activation. It also provides the substrate with a memory of its recent history, regarding which signalling pathways have been activated and that was the order of their activation.

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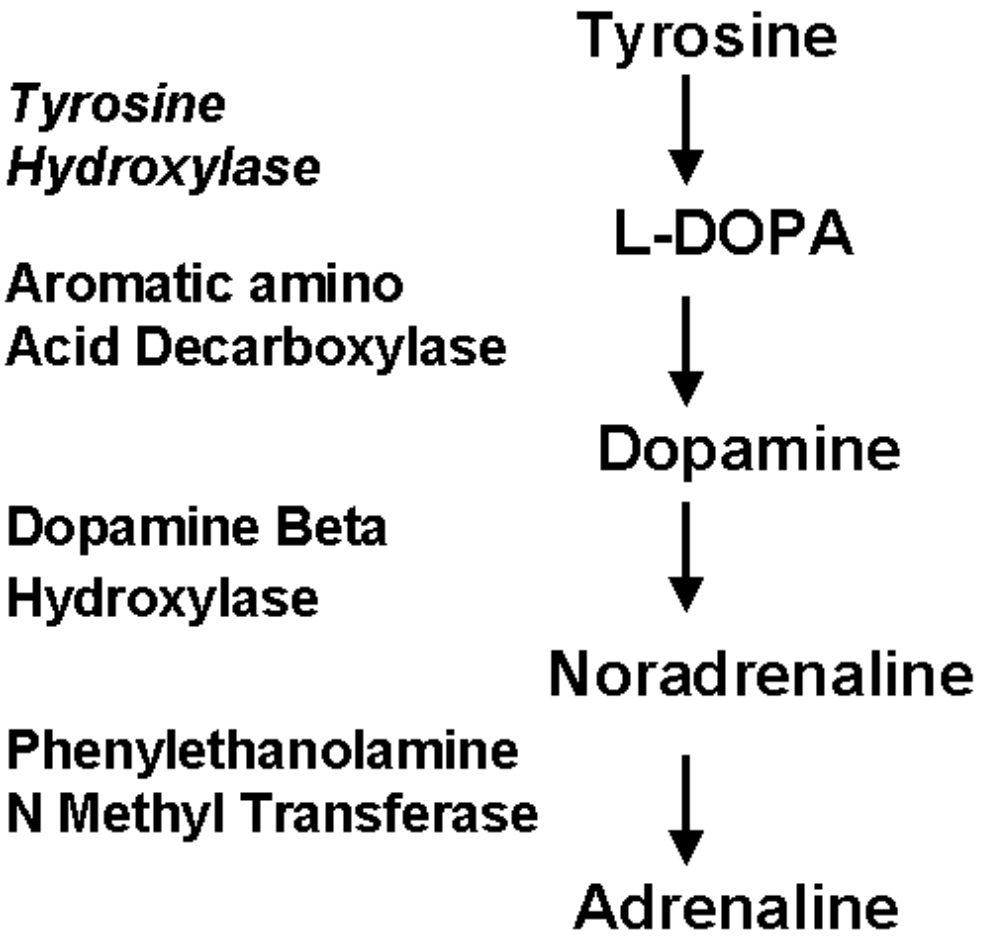
Tyrosine
Tyrosine Hydroxylase
L-DOPA
Aromatic amino Acid Decarboxylase
Dopamine
Dopamine Beta Hydroxylase
Noradrenaline
Phenylethanolamine N Methyl Transferase
Adrenaline

Fig. 1

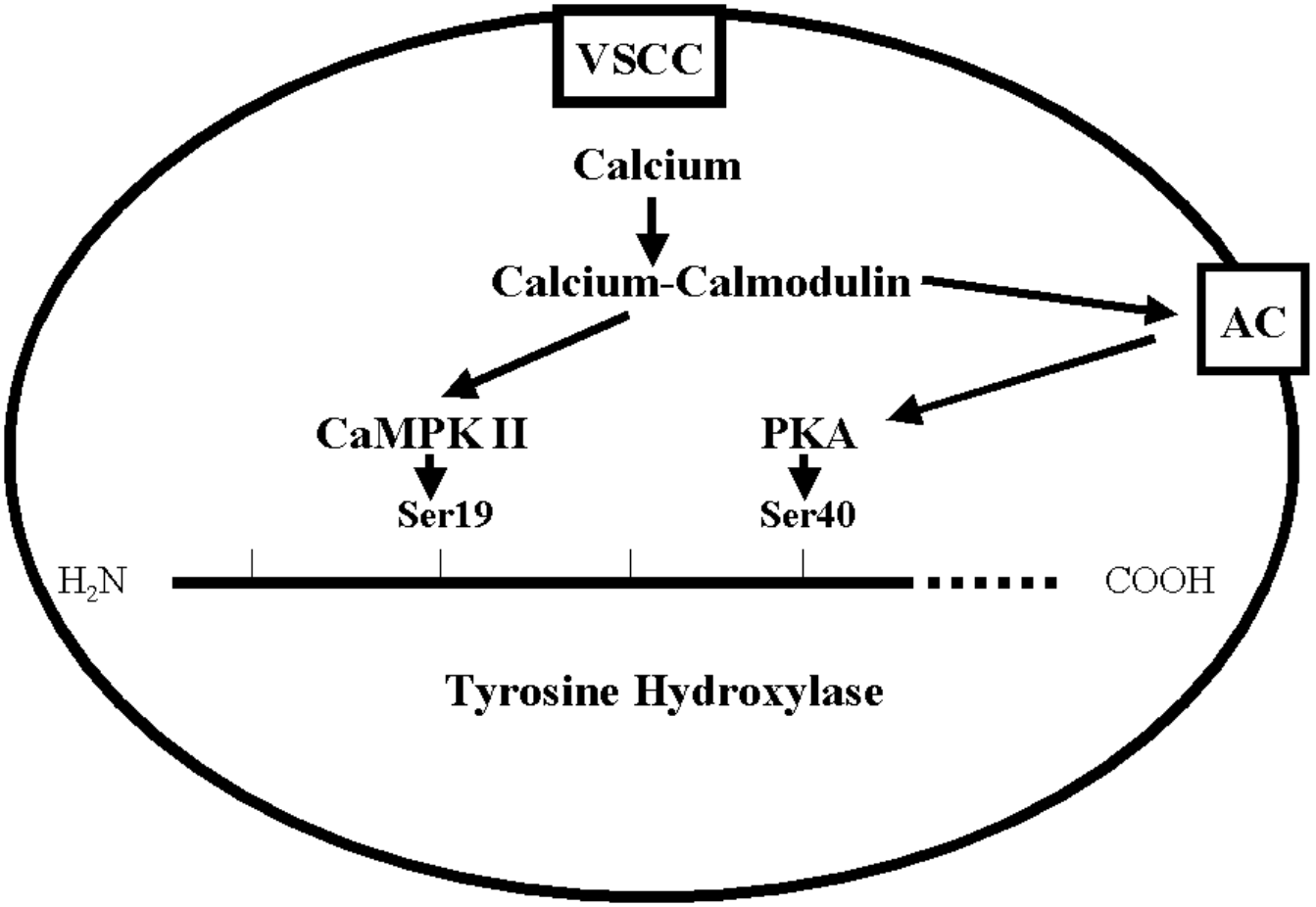
VSCC
Calcium
Calcium-Calmodulin
AC
CaMPK II
PKA
Ser19
Ser40
H2N
COOH
Tyrosine Hydroxylase

Fig. 2

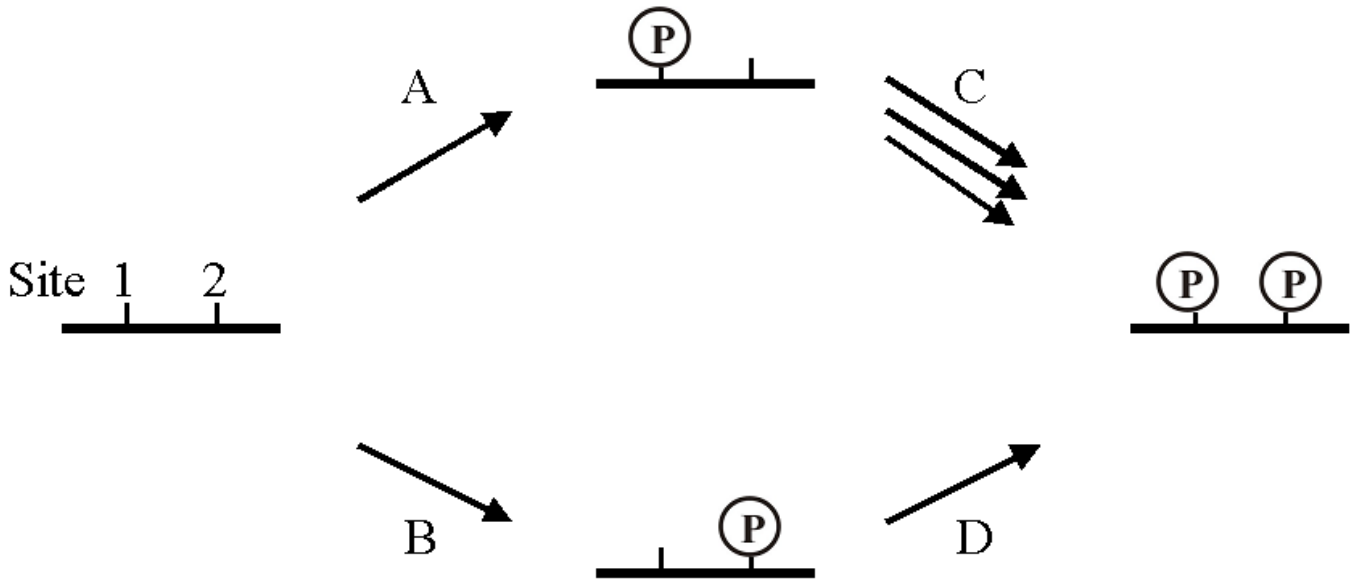

Fig. 3