Modelling and simulation of signal transductions in an apoptosis pathway by using timed Petri nets

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This paper first presents basic Petri net components representing molecular interactions and mechanisms of signalling pathways, and introduces a method to construct a Petri net model of a signalling pathway with these components. Then a simulation method of determining the delay time of transitions, by using timed Petri nets – i.e. the time taken in firing of each transition – is proposed based on some simple principles that the number of tokens flowed into a place is equivalent to the number of tokens flowed out. Finally, the availability of proposed method is confirmed by observing signalling transductions in biological pathways through simulation experiments of the apoptosis signalling pathways as an example.

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1. Introduction

Systems biology is a new field that aims to integrate different levels of information to understand how biological systems function. Generally in systems biology, mathematical modelling, simulation and analysis of biological systems play a critical role in helping biologists and biochemists explain and predict behaviour of a system.

Petri net is a formal description for modelling concurrent systems (Peterson 1981), and recently have been widely accepted as a description method for biological pathways by researchers in computer science as well as those in biochemistry (Pinney *et al* 2003). Various types of Petri net [e.g. low level Petri nets (Heiner *et al* 2001, 2004; Reddy *et al* 1993, 1996), stochastic Petri nets (Narahari *et al* 1989; Peccoud 1998), hybrid Petri nets (Matsuno *et al* 2003a,b), and coloured Petri nets (Genrich *et al* 2001)] have been widely applied to study metabolic pathways and signalling pathways in both quantitative and qualitative approaches because of potential advantages of Petri net possessing intuitive graphical representation and capabilities for mathematical analysis.

Metabolic pathways have such intrinsic characteristics that are series of chemical reactions catalyzed by enzymes, resulting in either the formation of a metabolic product to be used or stored by the cell, or the initiation of other metabolic pathways. The idea to use Petri nets for modelling and simulating metabolic pathways has been popularly

Keywords. Apoptosis; modelling; Petri net; signalling pathway; simulation.

Abbreviations used: Apaf-1, apoplectic protease activating factor 1; ATP, adenosine triphosphate; Bad, Bcl-x_L/Bcl-2 associated death promoter; Bax, Bcl-2 associated X protein; Bcl-2, B-Cell lymphoma 2; Bid, Bcl-2 interacting protein; caspase, cysteine-aspartic-acid-proteases; dATP, desoxyadenosine triphosphate; DED, death effector domain; DFF, DNA fragmentation factor; DFF40, 40 kDa unit of DFF; DFF45, 45 kDa unit of DFF; DISC, death inducing signalling complex; FADD, Fas-associated death domain protein; IGF-1, insulin-like growth factor-1; IL-1, Interleukin-1; IL-3, Interleukin-3; PDGF, platelet-derived growth factor; tBid, truncated Bid; TGF-beta, transforming growth factor-beta; TPO, Thrombopoietin; TNF, tumour necrosis factor;

developed (Hofestädt and Thelen 1998; Küffner *et al* 2000; Reddy *et al* 1993, 1996; Schuster *et al* 2000; Voss *et al* 2003; Zevedei-Oancea and Schuster 2003) owing to the simple mechanism that can be expressed by a uniform network of catalytic reactions.

In contrast, signalling pathways are generally more complex, consisting of distinct reactions such as complex formation, catalytic reaction, and translocation. With the feature of signalling pathways, a few researchers have tried to investigate relationships among complex molecular mechanisms and interactions, and further structural behaviours of signalling pathways by using Petri nets (Choi *et al* 2004; Heiner *et al* 2004; Lee *et al* 2004). In the paper (Heiner *et al* 2004), Heiner *et al* have explained how to model and validate the apoptosis pathways by using qualitative Petri nets. They have demonstrated a step-wise technique to model apoptosis signalling pathways. Further they have performed the model validation by using a standard Petri net analysis technique, and presented the biological meaning of the analysis results. Nevertheless, it is still expected to find a general methodology to model and analyse common signalling pathways by using Petri nets.

The current standard approach to represent biochemical reactions as a system is to use a series of ordinary differential equations (ODEs). This approach provides mathematically well-founded and fine interpretations of biological pathways. Accordingly, some trials have been made to model and simulate signalling pathways using ODEs (Hatakeyama *et al* 2003; Sasagawa *et al* 2005). Though ODE-based simulation can present quantitative behaviours of biological substances, it is hard to observe the whole system intuitively and grasp structural images of biological pathways from the series of differential equations. One approach to cope with this problem is to use hybrid Petri net (HPN) (Matsuno *et al* 2000, 2003a) which allows quantitative modelling and simulation of biological pathways with taking advantages of Petri net enabling graphical representation of biological pathways.

Quantitative analyses have also been made using discrete Petri net as found in the papers (Genrich *et al* 2001; Hofestädt and Thelen 1998; Popova-Zeugmann *et al* 2005). Popova-Zeugmann *et al* (2005) have introduced time Petri nets to bridge the gap between qualitative and quantitative models in steady state. They have presented structural techniques to decide the time-dependent realizability of a given transition sequence and to calculate its shortest and longest time length for the analysis of the time Petri net model.

In this paper, we propose a method of determining the delay time of transitions for firing by using timed Petri net models and perform simulations without establishing and tuning exact concentration. The paper is organized as follows. Firstly, we present a brief introduction of Petri net. Then we introduce a modeling method based on Petri net by taking notice of molecular interactions and mechanisms, and propose rules to determine the delay time of transitions. Further we show the Petri net model after explaining the biological background of Fas-induced apoptosis as an example. Finally, we use the example to demonstrate the usefulness of our modelling and simulation method by using a Petri net based simulation tool "cell illustrator" (Matsuno *et al* 2006).

2. Modelling signalling pathways with Petri nets

Petri nets are powerful tools in modelling and simulating various concurrent systems (Peterson 1981), especially biological pathways because Petri nets have the following superior characteristics (Matsuno *et al* 2006): (i) "firm mathematical foundation" enabling formal and clear description of biological pathways as well as their structural analysis; and (ii) "visual representation of networks" which provides intuitive understanding of biological pathways without any mathematical descriptions which are basically difficult for ordinary biologists.

In this section, we give the modelling rules for signalling pathways based on Petri net representation. The aims of the modelling by Petri net for signalling pathways are: (i) to make the biologists intuitively understand the intrinsic structure and features of signalling pathways; and (ii) to make it possible to mechanically model larger and more complicated signalling pathway networks.

2.1 *Basic definitions*

A Petri net N is defined by a 5-tuple $N= (T, P, E, \alpha, \beta)$ that corresponds to a bipartite graph, where T is a set of transitions represented by bars or boxes, P is a set of places represented by circles in a graph, E is a set of directed arcs between places and transitions, α denotes the weight of arc from place to transition, and β denotes the arc weight connected from transition to place. Note that the presence of multiple arcs is shown by a single arc with a non-zero positive integer arc weight.

A place can hold a positive integer number of tokens as its content. An assignment of tokens in each place expressed in form of a vector is called marking M, which varies during execution of a Petri net. A transition without

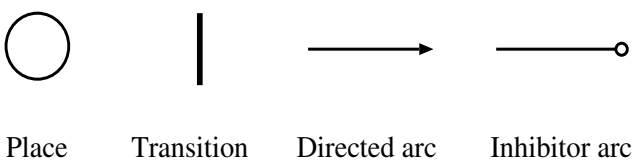


Figure 1. Basic elements of Petri net.

input places is called source transition that is always firable, and a transition without output places is called a sink transition likewise.

2.1a [*Firing rule of Petri nets* N]: A transition t is firable if each input place p_I of t has at least $\alpha_e(e = (p_I, t))$ tokens. A transition t fires to remove $\alpha_e(e = (p_I, t))$ tokens from each input place p_I and deposit $\beta_e(e = (t, p_O))$ tokens to each its output place p_O.

An inhibitor arc represents inhibitory function which is depicted as a line with a hollow circle at the end where the arrowhead normally appears. An inhibitor arc disables a transition to fire if the upstream place is occupied by a token, but does not consume the token. Figures 1 and 2 show basic elements of Petri net and the transition firing rules, respectively. For the details of Petri net theory, the readers are suggested to refer to Peterson (1981).

To do simulation of systems, Petri net model is usually extended by assigning firing time delay to transitions. Such extended Petri net is called timed Petri net $\overline{N} = (\overline{T}, \overline{P}, \overline{E}, \alpha, \beta)$. The firing rule of timed Petri net is extended as discussed below.

2.1b [*Firing rule of timed Petri nets* N]: (i) If the firing of a transition t_i is decided, tokens required for the firing are reserved. (ii) When the delay time d_i of transition t_i passed, t_i fires to remove the reserved tokens from the input place of t_i and put tokens into the output places of t_i.

In a timed Petri net, the firing times per unit time f_i, called firing frequency, of a transition t_i is constrained by its delay time d_i, and the maximum of firing frequency is the reciprocal of d_i.

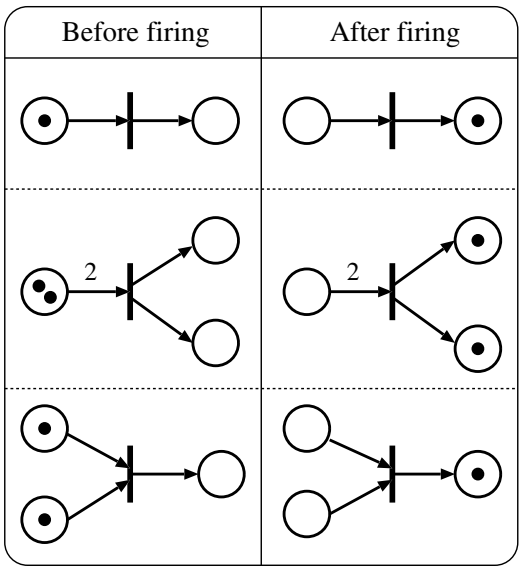


Figure 2. Examples show firing rules of Petri net.

2.2 *Modelling rules*

Here, we give the modelling method for signalling pathways with Petri net that can be naturally and explicitly modelled according to rules given below.

(i) Places denote static elements including chemical compounds, conditions, states, substances and cellular organelles participating in the biological pathways. Tokens indicate the presence of these elements. The number of tokens is given to represent the amount of chemical substances.

(ii) Transitions denote active elements including chemical reactions, events, actions, conversions and catalyzed reactions. A transition fires by taking off tokens from its individual input places and creating new tokens that are distributed to its output places if its input places has at least as many tokens in it as arc weight from the place to the transition.

(iii) Directed arcs connecting the places and the transitions represent the relations between corresponding static elements and active elements. Arc weights α and β describe the quantities of substances required before and after reaction, respectively. Especially in case of modelling a chemical reaction, arc weights represent quantities given by stoichiometric equations of the reaction itself. Note that, weight of an arc is omitted if the weight is 1.

(iv) An inhibition function in biological pathways is modelled by an inhibitor arc.

In cell biology, signalling pathways have been widely studied. They are information cascades of enzyme reactions from transmembrane receptors to the nucleus DNA, which ultimately regulate intracellular responses such as programmed cellular proliferation, gene expression, differentiation, secretion and apoptosis. Numerous reaction types of molecular interaction mechanisms have been described by Petri net model (Hofestädt 1994), which suffices to give the description of the metabolic pathway presently (Reddy *et al* 1993). For signalling pathways, as has been pointed (Takai-Igarashi and Mizoguchi 2004), besides the catalytic reactions, the information among the molecular interactions such as complex formation, gathering action, translocation and channel switching, also need to be respectively modelled by Petri nets according to different types of interactions as long as the biological facts has been known.

To explicitly understand the structural complicated signalling pathways, the modelling of each essential molecular interaction by using Petri net is the first step in modelling the network of signalling pathways as a qualitative event system. With a focus on possible molecular interactions as long as we have known, we summarize various molecular interactions of signalling pathways (see

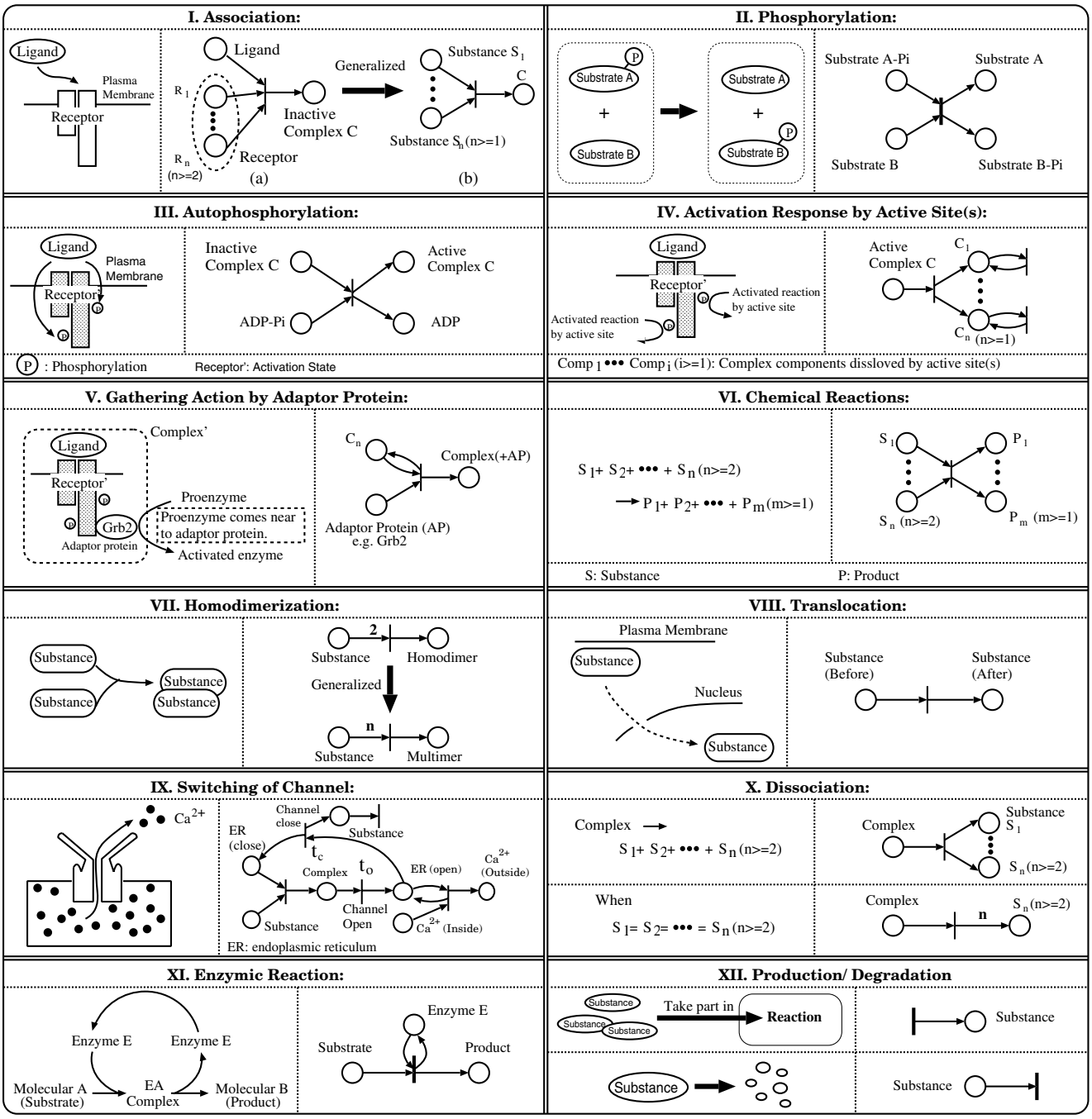


Figure 3. Petri net models of various reaction types in signalling pathways.

left side of dashed line in figure 3) and their corresponding Petri net model (right side of dashed line). Both of them in a reaction type are described as a 'block' labelled with roman numeral in this paper. Given below is the description of each molecular interaction and the corresponding model.

(I) *Association reaction*: It is a binding reaction to induce the formation of homo- or heterodimers and to generate a complex compound. This block shows the ligand-receptor binding interaction and corresponding Petri net model that indicates the transition is unfirable in the absence of place of ligand although receptors exist. The number of input place of transitions is two or more while the output place number is one in association reaction. Obviously, we also can expand the conception of association to the formation of model represented in block I (b), generally representing

the simultaneous association of substrates S_1, …, S_n ($n \geq 1$) forming a complex C in biological systems.

(II) *Phosphorylation*: It is a reaction to add a phosphate (PO_4) group to a protein or a small molecule, and dephosphorylation that is the backward reaction of phosphorylation removing phosphate groups from a compound by hydrolysis.

(III) *Autophosphorylation*: It is a transphosphorylation reaction frequently following the binding of a ligand to a receptor with intrinsic protein kinase activity.

(IV) *Activation response by active site(s)*: Generally a continued activated ligand-receptor complex regulates varied majority of cellular pathways transmitting the signals within the cell. Few methods using Petri nets have been proposed to model such activated complex place possessing more than one transition that can trigger down-stream signalling pathways (Heiner *et al* 2004). Their methods are easily understood, but have some problems that, if the transition of such place fires to remove the token(s) in shared input place at one time epoch, it will disable rest transitions simultaneously although the token will return back the same input via a self-loop. Hence, we need a more appropriate model to express this system's behaviour. Our basic consideration is that, if there have plurality of successive signalling pathways depending on distinct active site(s) (subunits) of activated complex, all the active site(s) shall be regarded as complex component(s) C_1, …, C_n ($n \geq 1$) as shown in block IV.

(V) *Gathering action by adaptor protein*: It is distinguished from association reaction. The main participator adaptor protein is an accessory protein to main proteins. These proteins lack the intrinsic enzymic activities themselves but instead mediate specific protein-protein interactions driving the formation of protein complexes.

(VI) *Chemical reactions*: It is the most common reaction in signalling pathways, which the conversion of substances to products is ordinarily modelled as input places to output places, both belonging to the same transition.

(VII) *Homodimerization*: It is a polymerization reaction of two identical substances to shape a dimmer similar to a kind of association reaction. A substance is modelled as an input place connected with a 2-weighted arc. It is easy to expand the conception to model the formation of multimer holding *n*-weight such as trimer and tetramer that is a complex of two or more equivalent polypeptides. The lower Petri net in block X is constructed under the opposite consideration of modelling homodimerization reactions.

(VIII) *Translocation*: It refers to the movement of molecules, substances or ions across cell membranes or via the bloodstream in biology. Figure 3 shows the nuclear translocation within a cell. A transition is modelled to indicate the movement action of substances before and after.

(IX) *Switching of channels*: Intracellular signal pathways are largely carried out by second messenger molecules. Ca^{2+} acts as a second messenger molecule to carry out large intracellular signal inside the cell. Usually the concentration of free Ca^{2+} within the cell is very low; it is stored inside of organelles, mostly the endoplasmic reticulum. In order to become active, Ca^{2+} has to be released from the organelles into the cytosol. Two transitions t_o and t_c are introduced to denote channel activity of 'open' and 'close', respectively. t_o is enabled when input place holds up token(s) after the association of organelles and substances, whereas t_c is enabled as long as some stop mechanisms shutoff the channel.

(X) *Dissociation*: This is the opposite of I. Dissociation process is a general process in which complexes and molecules separate or split into smaller molecules, ions. The number of input place of transitions is one while the output place number is two or more.

(XI) *Enzymic reaction*: Since an enzyme itself plays a role of catalyzer in biological pathways and there occurs no consumption in biochemical reactions, the reaction is modelled to a transition, whereas the enzyme is modelled to enzyme place that has a self-loop with same arc-weight. That is, once an enzyme place is occupied by a token, the token will return to the place again to keep the firable state, if the transition is fired.

(XII) "*Production Degradation*": A source transition represents an activity to provide substances that will take part in the reactions. A sink transition denotes small and natural degradation of substance.

3. Modelling and simulation of signalling pathways

In this section, we use the example of apoptosis to demonstrate our modelling method and then explain how to simulate.

3.1 *Biology background and modelling of apoptosis*

Apoptosis – a form of cell death characterized by cell shrinkage, membrane blebbing, nuclear breakdown, and DNA fragmentation – is a vital cell lifecycle decision point for development, maintenance of tissue homeostasis, and elimination of harmful cells in metazoan organisms (Jacobson *et al* 1997; Nagata 1997). Caspases (cysteine-aspartic-acid-proteases) are a group of cysteine proteases existing as inactive zymogens that can be cleaved by other proteins within the cell resulting in the apoptotic process. Failure of apoptosis is one of the main contributions to tumour development and autoimmune diseases such as neurodegenerative diseases, AIDS and ischemic stroke (Thompson 1995). Different cellular signals can initiate

activation of apoptosis on different ways in dependence of the various kinds and biological states of cells. Fas-induced apoptosis has been studied in detail and its mechanism has been proposed. Fas ligand is a type II transmembrane protein belonging to tumour necrosis factor (TNF) family, which signals apoptotic effects to nucleus through several major pathways as shown in figure 4.

Fas ligands, existing as trimers, bind to Fas receptor and promote receptor trimerization. Adaptor proteins Fas-associated death domain protein (FADD) in turn associate with the receptors through an interaction between homologous death domain (DD) on the receptor and FADD. Furthermore, FADD contains death effector domain (DED) that allows binding of procaspase-8 to the receptor complex to form a death inducing signalling complex (DISC) (Scaffidi *et al* 1998; Weber and Vincenz 2001). Upon the association with FADD through DED, procaspase-8 is autocatalytically activated to produce caspase-8. The activation of caspase-8 initiates the following two pathways leading to the activation of downstream caspases.

(1) caspase-8 activates downstream caspases indirectly by cleaving B-cell lymphoma 2 (Bcl-2) interacting protein (Bid) and COOH-terminal part of Bid (tBid) translocates onto mitochondria (Mit for short) where it makes cytochrome c leak out and enter the cytosol. Released cytochrome c binds to apoplectic protease activating factor-1 (Apaf-1) together with dATP/ATP and procaspase-9 and achieve the caspase-9 activation based on three-step reactions as illustrated in figure 4 (Hu *et al* 1999; Li *et al* 1997; Zou *et al* 1999): (i) dATP/ATP binds to Apaf-1 and is hydrolyzed to dADP or ADP, respectively; (ii) cytochrome c binds to Apaf-1 and promotes the multimerization of Apaf-1/ cytochrome c complex, forming a so-called apoptosome made of at least eight subunits when dATP/ATP bound to Apaf-1 is hydrolyzed; and (iii) once apoptosome is formed, procaspase-9 is recruited to apoptosome, and it becomes activated through autocatalysis.

Activated caspase-9 releases from apoptosome to cleave downstream caspases such as caspase-3, and new procaspase-9 is activated through autocatalysis by either apoptosome or fresh activated caspase-9 (Luo *et al* 1998).

(2) caspase-8 activates downstream caspases such as caspases-3 by directly cleaving them.

Activated caspase-3 cleaves DNA fragmentation factor (DFF) composed of 45 kDa (DFF45) and 40 kDa (DFF40) subunits. Cleaved DFF45 dissociates from DFF40, accompanied by DFF40 homo-oligomer formation that possesses DNase activity (Saleh *et al* 1999; Widlak *et al* 2003). Finally, DFF40 oligomer induces DNA fragmentation and chromatin condensation that are regarded as apoptotic hallmarks.

Figure 5 shows the whole Petri net model of Fas-induced apoptosis based on our modelling rules. The modelling operation starts from the source transition t_1 denoting an activity that the substances take part in reactions. Here we only explain the case of the pathway whose caspases are directly cleaved by caspase-8 (p_{10}) to trigger DNA damage: Fas ligands and corresponding receptor are assigned to places (p_1 and p_3) connected from the source transitions (t_1 and t_3) respectively, in accordance with block XII of figure 3. Since three Fas ligands shape a Fas ligand trimer, a transition t_2 is used to represent polymerization connecting from the input place p_1 with a 3-weighted arc $e(p_1, t_2)$ as well as connecting to the output place p_2 of Fas ligands trimer applying the block VII (figure 3). Next, the ligand-receptor binding interaction occurs and corresponding Petri net model that two places (p_2 and p_3) merge into a place p_4 denoting ligand-receptor complex via the transition t_4 represents association reaction. Succedent reactions for the production of intermediate products: ligand-receptor/FADD complex (p_6) and DISC (p_8) are modelled in the same way using both of blocks I (figure 3), i.e. association reaction. procaspase-8 (p_7) contained in DISC (p_8) is autocatalytically cleaved to produce caspase-8 (p_{10}) which is modelled as a dissociation reaction X represented by t_9. Since caspase-8 (p_{10}) next initiates the activation of downstream caspases and there occurs no consumption in the reactions, caspase-8 (p_{10}) is modelled to enzyme place with a self-loop to take part in four enzymic reactions including two autocatalytic activations of new caspase-8, activation of caspase-3 cascades and mitochondrial DNA damage pathways. The reaction that activated caspase-3 catalyzes DFF (p_{34}) to the productions of DFF40 and DFF45, is modelled as an enzymic reaction represented by t_{42}. DFF40 further form a DFF40 homo-oligomer (p_{39}) which applies the reaction model of homodimerization as shown in block VII (figure 3) represented by t_{44}. At last, the transition t_{49} is used to model the enzymic reaction where DFF40 oligomer acts as DNase to induce DNA fragmentation whose corresponding place p_{41} connects to sink transition t_{51} indicating the natural degradation to the system environment (see block XI and XII, figure 3).

In this way, modelling of apoptosis can be done by connecting the reaction types listed in figure 3. The biological interpretation for each transition used in this model is summarized in table 1. Note that this model is of no delay time for transitions.

3.2 *Creating timed Petri net for pathway simulation*

As described above, the Petri net model illustrated in figure 5 has been drawn. This model describes the structure of connection relation. The next task we should do is to confirm the validity of the model, that is whether proposed models are in accord with the biological facts of reactions or not. Therefore, we consider a new simulation method by which

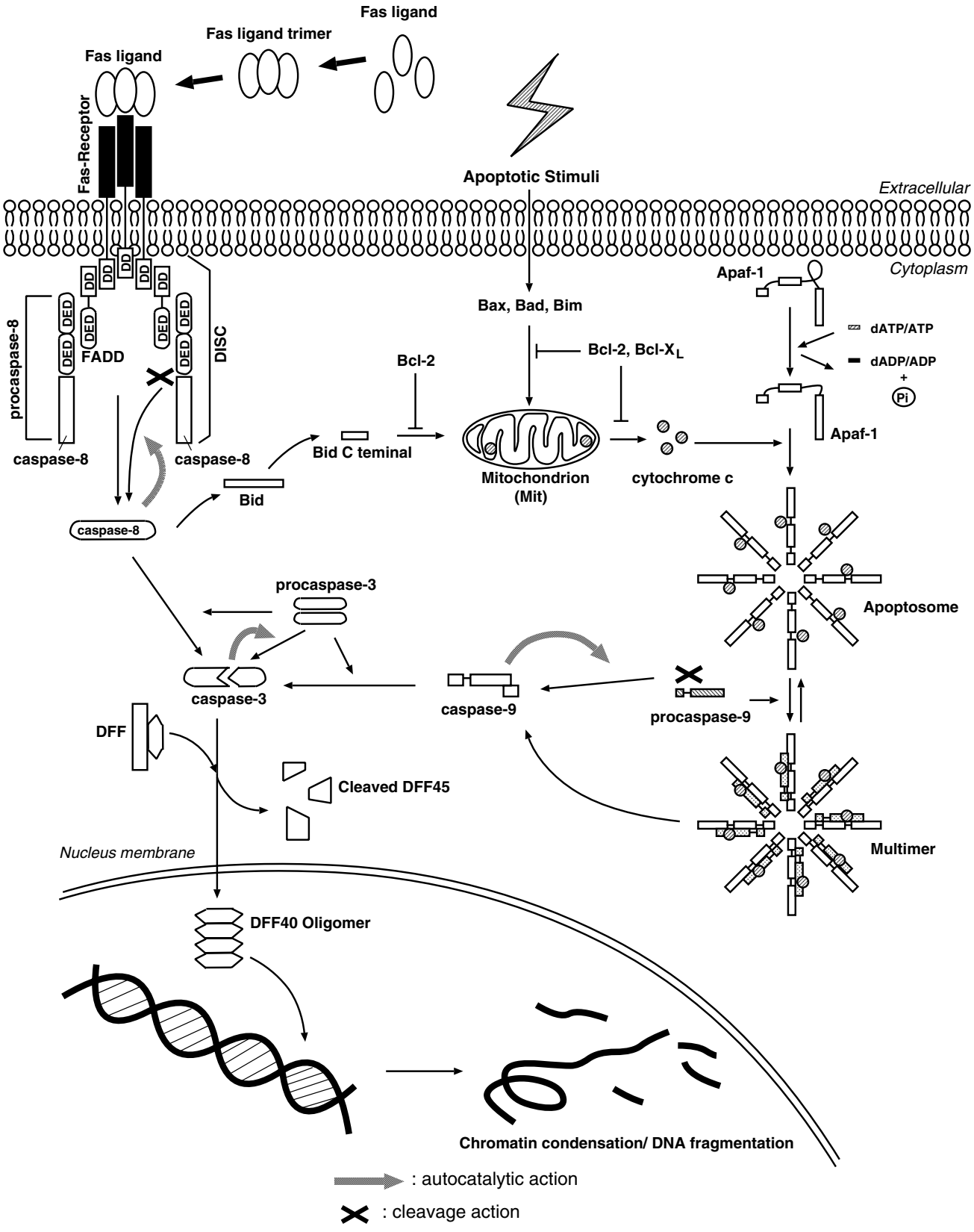


Figure 4. Biological diagram of apoptosis pathways induced by Fas ligands.

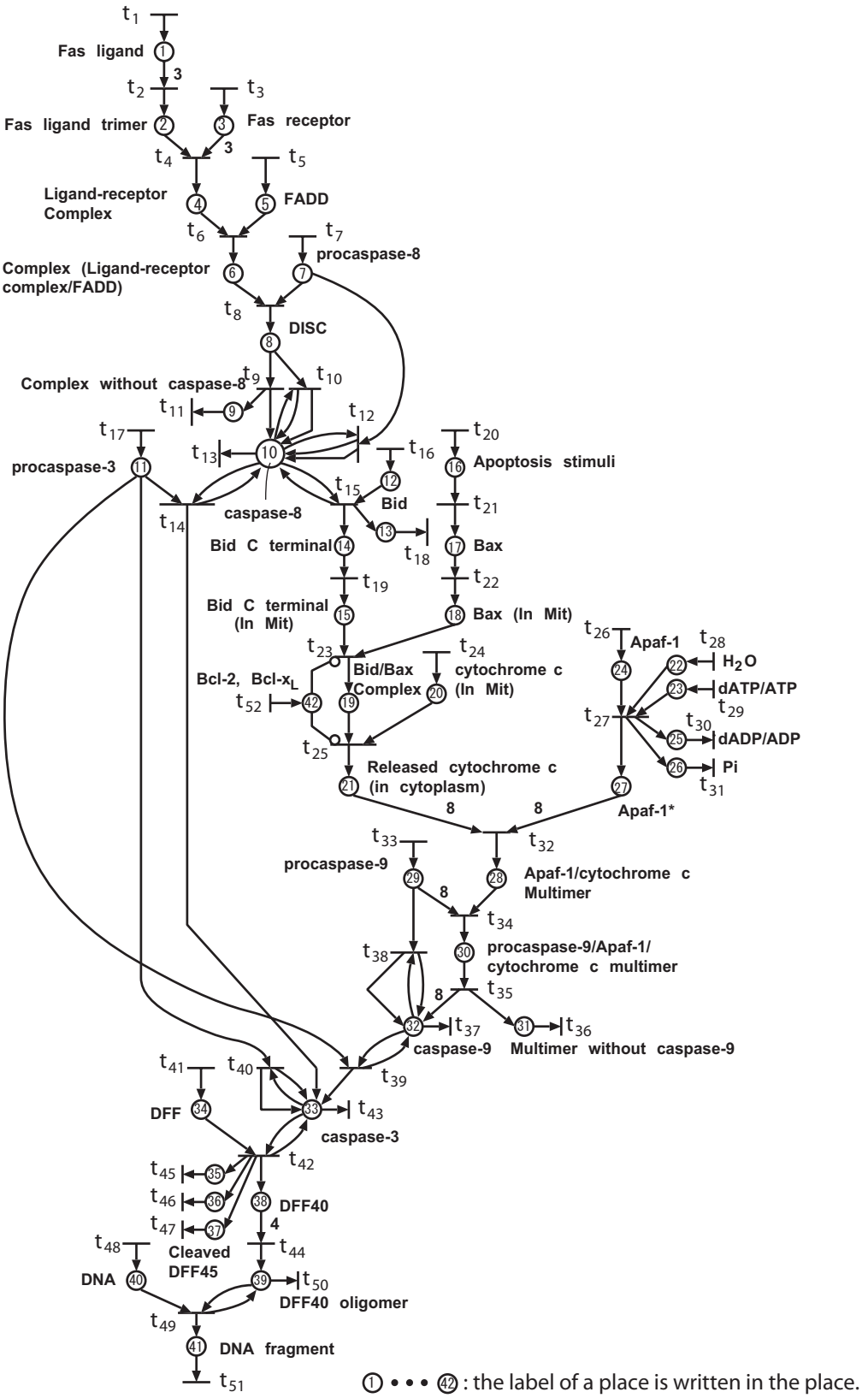


Figure 5. Petri net model without time of figure 4.

Table 1. Biological interpretation of each transition in figure 5.

Transition	Reaction type	Biological interpretation
t_1, t_3, t_5, t_7, t_{16}, t_{17}, t_{20}, t_{24}, t_{26}, t_{28}, t_{29}, t_{33}, t_{41}, t_{48}, t_{52}	XII. Production/Degradation	The substances represented by output places of transitions attend the reactions from system environment
t_2, t_{32}, t_{44}	VII. Homodimerization	Polymerization reaction of some identical substances to shape a multimer
t_4, t_6, t_8, t_{23}, t_{34}	I. Association	Association reaction with the binding to induce the formation of a complex
t_9, t_{35}	X. Dissociation	Complexes, substances separate or split into smaller molecules
t_{10}, t_{12}, t_{14}, t_{15}, t_{38}, t_{39}, t_{40}, t_{42}, t_{49}	XI. Enzymic reaction	Substrates are catalyzed to the productions; there occurs no consumption in biochemical reactions
t_{11}, t_{13}, t_{18}, t_{30}, t_{31}, t_{36}, t_{37}, t_{43}, t_{45}, t_{46}, t_{47}, t_{50}, t_{51}	XII. Production/Degradation	Natural degradation of substances
t_{19}, t_{22}	VIII. Translocation	The movement action of substances from cytoplasm to mitochondria membranes
t_{21}, t_{25}, t_{27}	VI. Chemical reaction	The conversion of substances to products by a chemical reaction

(i) the traces of signal transductions can be easily understood and (ii) the transduction speeds of each pathway leading to cell death can be observed.

For controlling the transduction speeds of signals in a pathway, each transition in the Petri net model should have delay time reflecting the speed of corresponding biological reactions. Basic facts for deciding delay time can be obtained from biological experiments and scientific common principles. However, in the majority of cases, reliable data of detailed reactions have not been reported in biological literature.

These observations lead us to develop a new method that determines transition speeds with which token flows in the modelled Petri net represent the signal transductions in the original signalling pathways.

3.2a *Basic principles*: (1) The sum of consumption is equal to the production to keep the concentration equilibrium for each substance engaged in signalling pathways, i.e. for each place the token amounts flowed-in and flowed-out per unit time are equivalent.

$$\sum_{i=1}^{m} K_{I_i} = \sum_{j=1}^{n} K_{O_j};$$

where, $\sum_{i=1}^{m} K_{I_i}$ and $\sum_{j=1}^{n} K_{O_j}$ are the total token amounts flowed-in and flowed-out per unit time, respectively (figure 6). (2) As defined in § 2.1, delay time d_i is the reciprocal of the maximum of firing frequency f_i, and it is obvious that the token amounts flowed-in and flowed-out for each place per unit time are expected to be kept equivalent under the fastest firing frequency f_i. Therefore, in this paper we decide d_i by calculating f_i.

Note that, in this paper we suppose that apoptosis pathway is such a biological system that if required

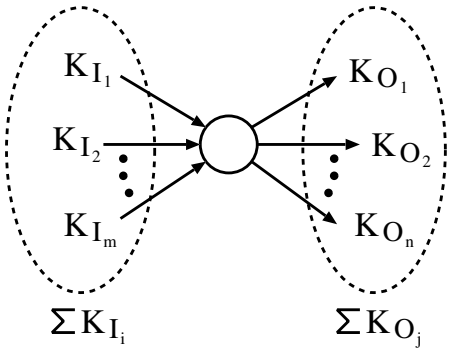


Figure 6. Illustration for Basic principles, (1).

substances are assembled, the reactions promptly become possible. Thus, what we have to discuss is how to assign the delay time to each transition in timed Petri net $\overline{N}$. In the following, we give such delay time determination rules for each transition.

3.2b *Strategy for determining transition speeds:* Rule (1): If there is a place p_i that the number of input transitions is one or more while the number of output is one, the maximum of firing frequencies $\{f_i\}$ satisfy the following equation: i.e.;

$$\sum_{i=1}^{m} \beta_i \cdot f_{I_i} = \alpha \cdot f_O,$$

where α and β_i are the weights of arcs $e(p_i, t_O)$ and $e(t_{I_i}, p_i)$, respectively. t_{I_i} and t_O are the input and output transitions of place p_i. Furthermore, f_{I_i}, f_O are the maximum of firing frequencies of t_{I_i}, t_O, respectively (figure 7).

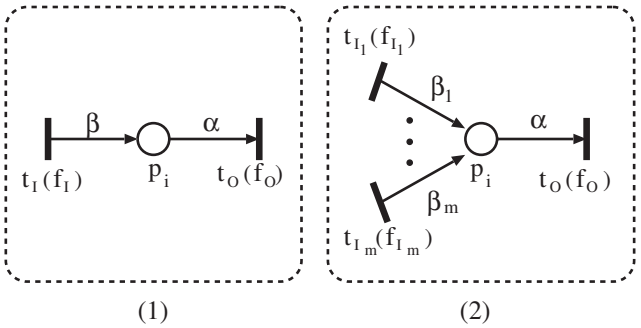


Figure 7. Two connection cases applied to rule (1).

Rule (2): If there is a place p_i whose output transitions are in conflict, the maximum of firing frequencies $\{f_i\}$ satisfy following linear equation and inequality represented as:

$$\begin{cases} \sum_{i=1}^{m} \beta_i \cdot f_{I_i} = \sum_{j=1}^{n} \alpha_j \cdot f_{O_j} \\ 2 \cdot \frac{f_{O_n}}{\alpha_n} \geq \frac{f_{O_1}}{\alpha_1} \geq \frac{f_{O_2}}{\alpha_2} \geq \ldots \geq \frac{f_{O_n}}{\alpha_n} \text{ ; and} \end{cases}$$

where α_j and β_i are the weights of $e(p_i, t_{O_j})$ and $e(t_{I_i}, p_i)$, respectively, and α_j satisfies $\alpha_1 \geq \alpha_2 \geq \ldots \geq \alpha_n$. t_{I_i}, t_{O_j} are the input and output transitions of place p_i, and f_{I_i}, f_{O_j} are the maximum of firing frequencies of t_{I_i}, and t_{O_j}, respectively as shown in figure 8.

Rule (3): Use a small value as the maximum of firing frequencies to sink transition connected from the enzyme place.

In the above rule (1), the equation is applied to a place that has a single output transition. Obviously, this single transition can fire smoothly only according to the delay time. However, in apoptosis Petri net model, there exist situations that transitions (e.g. t_{14}, t_{39} and t_{40}) are in conflict since firing of transition will remove the token from the input place disabling the other transitions. As we have stated above, biological experiments for measuring such reactions have not been executed yet in majority of cases. Therefore, in this paper we assume that such enabled transitions in conflict have the same chance to fire. Under this assumption, the first equation of rule (2) is designed to obey basic principle (1), and the second inequality is considered for two reasons: (i) The right-hand member $\frac{f_{O_1}}{\alpha_1} \geq \frac{f_{O_2}}{\alpha_2} \geq \cdots \geq \frac{f_{O_n}}{\alpha_n}$ is designed to guarantee the firing of t_{O1} with the maximum arc-weight α_1 such that t_{Oi} do not fire later than the firing of t_{On} with minimum arc-weight α_n. (ii) The left-hand member $2 \cdot \frac{f_{O_n}}{\alpha_n} \geq \frac{f_{O_1}}{\alpha_1}$ is in order to make the transition t_{Oi} not fire too fast than the t_{On} that has the minimum arc-weight (make the first firing of t_{On} earlier than the second firing of t_{Oi} to be exactly). Rule (3) is designed to express the moderately slow and small natural degradation of enzymes.

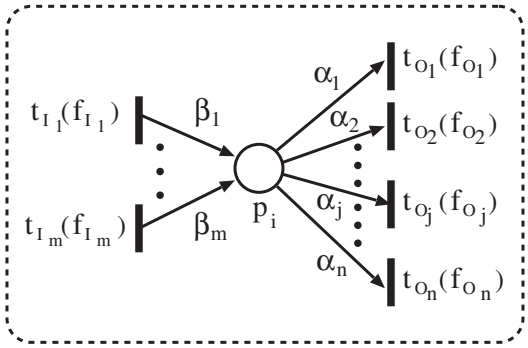


Figure 8. Conflict situation applied to rule (2).

Furthermore, when using timed Petri net to simulate the apoptosis, the self-loops of enzyme places are replaced by test arcs with threshold due to the attribute that a test arc does not consume any content of the place at the source of the arc by firing. The firing rules are defined as: (i) When the value of threshold equals 1, nothing need to be done since the transition at the sink of test arc can fire constantly as long as the place at the source of test arc is occupied by tokens; and (ii) when the value of threshold is more than 1, the test arc is handled as general arc and the transition at its sink has to comply with the above rules in order to guarantee the firing of the transition connected from the test arc.

In the following, we demonstrate how our model is practically executed (simulated) and the explanation of several transitions applying corresponding rules is detailedly given as an example. Furthermore, applying timed Petri net model allows to replace the initial marking instead of the source transitions, because of the facts that survival cells receive a certain number of extracellular stimuli and the amount of substances involved in corresponding reaction is not infinite (but enough many). And the number of initial tokens can be obtained. For example, the number of initial tokens for the place of Fas ligand equals $\alpha \cdot f_2 = 3 \cdot 1 = 3$ obtained by applying rule (1) due to its single output transition t_2. Note that, the delay time of the most apical transition is supposed to 1.

Figure 9 illustrates the timed Petri net model of apoptosis. In figure 9, the place of Fas ligand trimer is such a place that the number of input and output transitions is respectively one, so that the maximum of firing frequencies f_4 satisfy the equation of rule (1), i.e. $\beta \cdot f_2 = \alpha \cdot f_4$, where, since $\beta = \alpha = 1$, and $f_2 = 1$, then $f_4 = 1$ is obtained. In calculating the delay time of t_9 and t_{10}, we apply rule (2) because they are in conflict. Based on the equation of $\beta \cdot f_8 = \alpha_1 \cdot f_9 + \alpha_2 \cdot f_{10}$, since $\beta = \alpha_1 = \alpha_2 = 1$, $f_8 = 1$, f_9 and f_{10} are assumed to have the same firing chance, $f_9 = f_{10} = 1/2$ are obtained and accordingly $d_9 = d_{10} = 2$ are obtained. That is, the transition t_9 and t_{10} have the

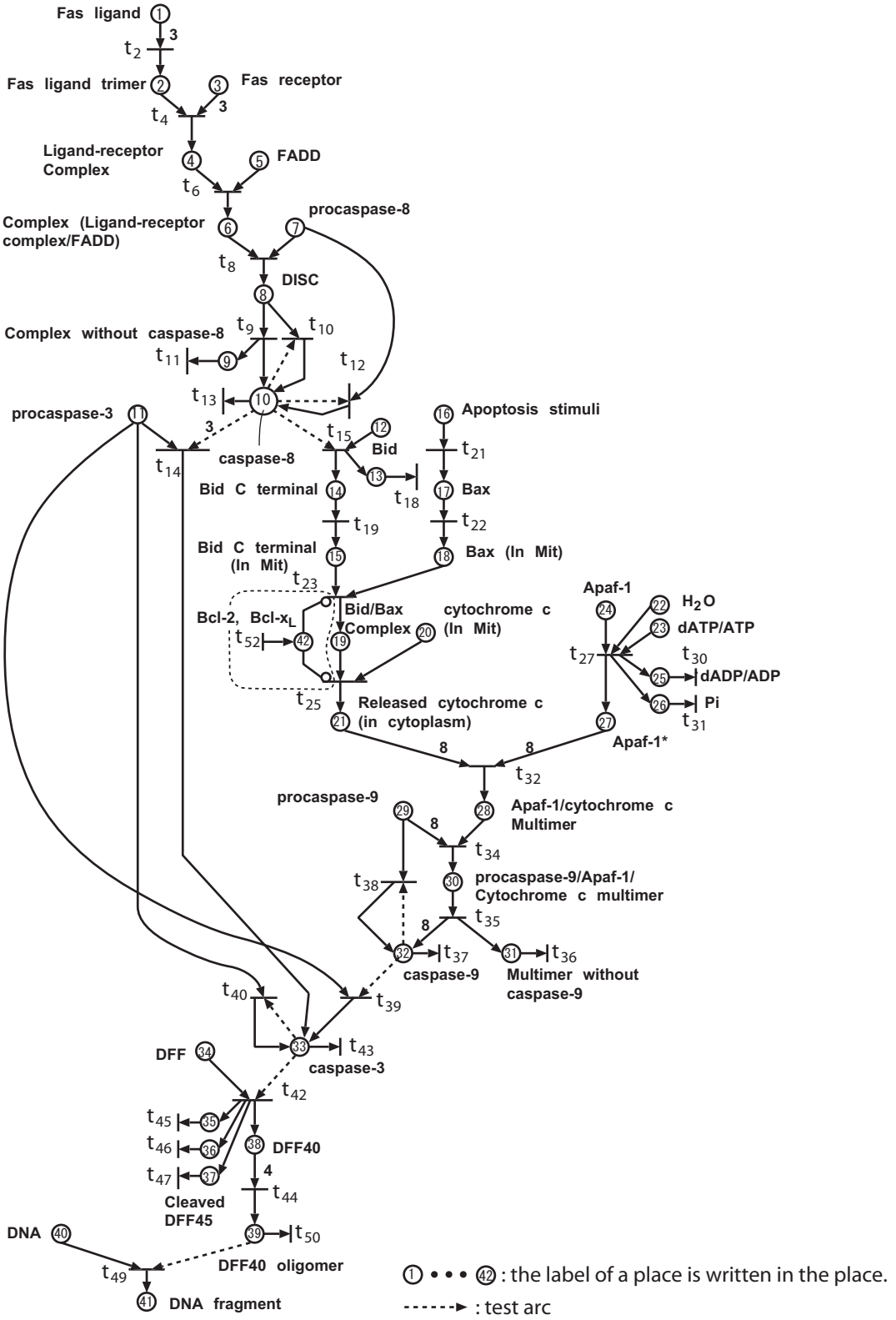


Figure 9. Timed Petri net model of apoptosis based on figure 5. The block surrounded by dashed lines does not take part in the simulation of timed Petri net models because of the assumption that apoptosis pathways propagate the 'signal' without receiving the signals from any signal pathway having a relation to t_{52} when simulating the timed Petri net.

equal chance to fire after d_9 or d_{10}. Note that β is the arc-weight of $e(t_8, p_8)$. α_1 and α_2 are the arc-weight of $e(p_8,t_9)$ and $e(p_8,t_{10})$, respectively. The delay time d_{37} of the transition t_{37} is assigned to 10 as an example that is based on rule (3) due to the feature of small and natural degradation of caspase-9.

4. Simulation results and discussions

With the processes to decide the delay time of transitions to timed Petri net model in § 3.2, the delay time for transitions can be determined and the number of initial tokens of places can be given as listed in tables 2 and 3. The constructed timed Petri net model (figure 9) of apoptosis is simulated by using Cell Illustrator with the decided delay time and the number of initial tokens.

In the Petri net model as shown in figure 5, the production of Bcl-2/BCL-x_L is generally influenced by the activities of other signalling pathways, thus the place p_{42} of Bcl-2/BCL-x_L and input transition t_{52} are specially adopted to represent these activities. However, the apoptosis pathway of this paper is supposed to propagate the 'signal' without receiving the signals from any signal pathway having a relation to t_{52} when simulating the timed Petri net. That is, the transition t_{52} does not fire. Therefore, the simulation of timed Petri net models can be performed without inhibitor arcs.

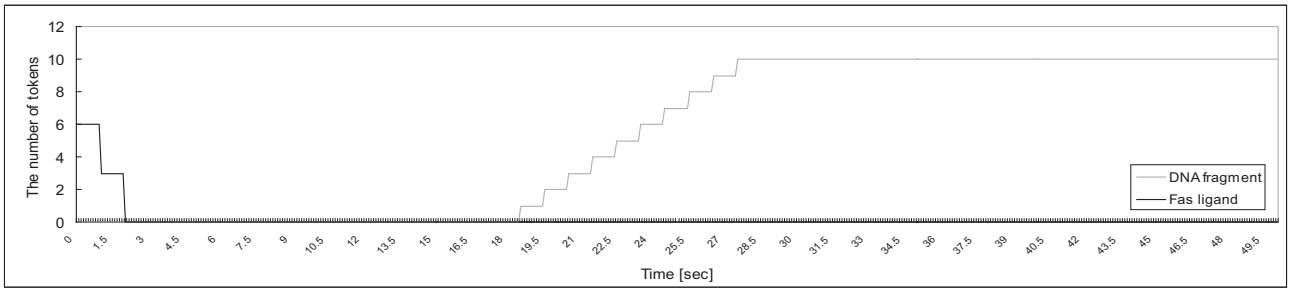
The simulation results are presented in figure 10. (1) The response of DNA fragments to Fas ligands is expressed as the token behaviours in figure 10 (a): Fas ligand concentration reaches zero at the time 2.0 (s), and DNA fragments that is often considered as an indicator of cell death, start to increase after the time 19.0 (s). Apoptotic pathways are working to propagate signals from Fas ligands

Table 2. The number of initial tokens for the output places of source transitions.

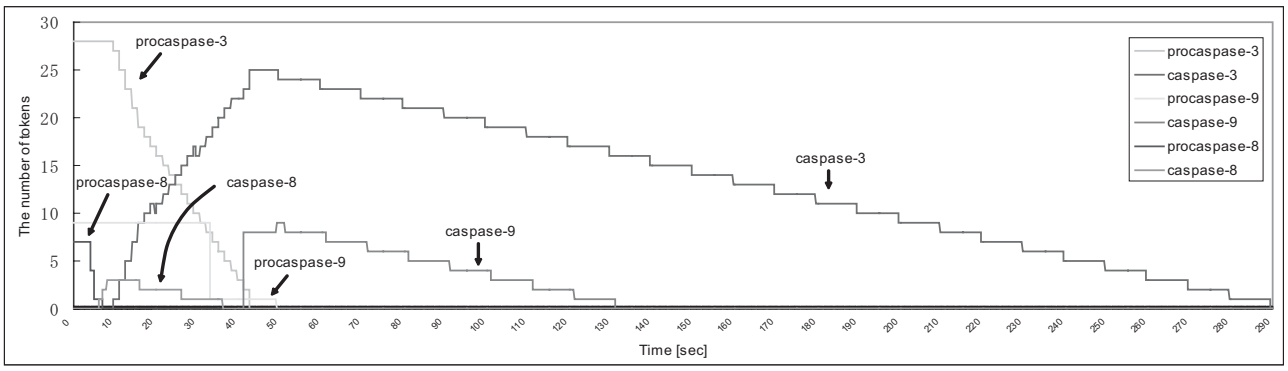
Place p_i	Biological substance	Initial tokens
p_1	Fas ligand	6
p_3	Fas receptor	6
p_5	FADD	6
p_7	procaspase-8	7
p_{11}	procaspase-3	28
p_{12}	Bid	8
p_{16}	Apoptosis stimuli	8
p_{20}	cytochrome c	8
p_{22}	H_2O	8
p_{23}	dATP/ATP	8
p_{24}	Apaf-1	8
p_{29}	procaspase-9	9
p_{34}	DFF	4
p_{40}	DNA	10

Table 3. Transition speeds in timed Petri net model for apoptosis illustrated in figure 9.

Transition t_i	Reaction type	Delay time d_i (s)	Transition t_i	Reaction type	Delay time d_i (s)
t_2	VII. Homodimerization	1	t_{30}	XII. Degradation	1
t_4	I. Association	1	t_{31}	XII. Degradation	1
t_6	I. Association	1	t_{32}	VII. Homodimerization	8
t_8	I. Association	1	t_{34}	I. Association	8
t_9	X. Dissociation	2	t_{35}	X. Dissociation	8
t_{10}	XI. Enzymic reaction	2	t_{36}	XII. Degradation	8
t_{11}	XII. Degradation	2	t_{37}	XII. Degradation	10
t_{12}	XI. Enzymic reaction	1	t_{38}	XI. Enzymic reaction	8
t_{13}	XII. Degradation	10	t_{39}	XI. Enzymic reaction	1.5
t_{14}	XI. Enzymic reaction	1.5	t_{40}	XI. Enzymic reaction	1.5
t_{15}	XI. Enzymic reaction	1	t_{42}	XI. Enzymic reaction	1
t_{18}	XII. Degradation	1	t_{43}	XII. Degradation	10
t_{19}	VIII. Translocation	1	t_{44}	VII. Homodimerization	4
t_{21}	VI. Chemical reaction	1	t_{45}	XII. Degradation	1
t_{22}	VIII. Translocation	1	t_{46}	XII. Degradation	1
t_{23}	I. Association	1	t_{47}	XII. Degradation	1
t_{25}	VI. Chemical reaction	1	t_{49}	XI. Enzymic reaction	1
t_{27}	VI. Chemical reaction	1	t_{50}	XII. Degradation	10



(a) Simulation results of Fas ligand and DNA fragmentation



(b) Simulation results of procaspases and caspases

Figure 10. Simulation results of timed Petri net model of apoptosis in figure 9. **(a)** Token numbers representing DNA fragments increase in response to the initial amount of tokens of Fas ligands. **(b)** Concentration behaviours of (pro-)caspases working in the apoptotic pathway. The order of expression of the caspases in this graph is exactly the same as the order of them in Matsuno *et al* (2003a), which is obtained by manual tuning of transitions in the apoptotic pathway.

to the DNA. (2) The diagram (b) shows the number of tokens and further the activation order of three kinds of caspases involved in apoptosis pathways: (i) caspase-8 is activated ahead of caspase-3 and caspase-9; (ii) caspase-3 is activated afterwards which is activated directly by caspase-8 and the amount of caspase-3 has a notable augment that can be observed as the result of the autocatalytic activation; and (iii) caspase-9 begins to be activated after a short time from the activation of caspase-3. This is due to the activation of caspase-9 which relies on the mitochondrial DNA damage pathways that include the reactions such as the release of cytochrome c from mitochondria, the formation of apoptosome and so on.

By comparing the behaviours of simulation results executed by Matsuno *et al* (2003a), we can observe: (i) both of the diagram (a) and (b) in figure 10 have the approximate same waveforms as the simulation results in Matsuno *et al* (2003a), i.e. the amount of DNA destruction is increased along with the decrease of Fas ligands due to the complex formation of Fas ligand trimer with the lapse of time; and (ii) the activation order of three kinds of caspases is caspase-8, caspase-3, caspase-9, which is exactly the same as the one derived from the simulation in Matsuno *et al* (2003a).

Since the simulation results reported by Matsuno *et al* (2003a) have been successfully obtained by using "hybrid Petri nets", our proposed simulation method may probably provide quite promising results based on timed Petri net model. However, hybrid Petri nets use continuous places and transitions. The reaction rates of transitions are assigned as real numbers and the parameters for initial concentration of substances and transition speeds are carefully tuned by hand. In general, lots of trail and error processes have been performed until appropriate parameters for simulation are determined. The simulation is precise and is executed in very small time interval. The parameter tuning processes consume too much time. Therefore, with the simulation results, the appropriateness of the timed Petri net model of apoptosis is verified that the simulation method may probably provide a number of valuable insights.

In addition, since Cell Illustrator possesses the excellent graphical user interface, the timed Petri net of apoptosis can be simulated in the animation method. In this way, the

different apoptotic pathways can be pursued by tracing the flow of tokens as represented below.

(1) Fas ligand induced pathway:
Fas ligand/Fas receptor/FADD/DISC/procaspase-8/caspase-8/procaspase-3/caspase-3 pathway.

(2) mitochondrial DNA damage pathway:
Fas ligand/Fas receptor/FADD/DISC/procaspase-8/caspase-8/Bid/Bax/cytochrome c/Apaf-1/apoptosome/caspase-9/caspase-3 pathway.

In contrast, the continuous Petri net models have no such feature to make the biologists intuitively understand the intrinsic structure and behavioural properties of signalling pathways by simulating the models.

5. Concluding remarks

We have first presented basic Petri net components representing molecular interactions of signalling pathways, and introduced a method to construct a Petri net model of a signalling pathway with these components. Then we have described a method of determining transition speeds of a Petri net based on some simple principles that the number of tokens flowed into a place is equivalent to the number of tokens flowed out of the place. Finally we have confirmed the availability of proposed method through the modelling and simulation of the apoptosis signalling pathway. The main contributions are: (i) to give a consistent description of signalling pathways with the Petri net models of molecular interactions and mechanisms in figure 3, which enables not only biologists but also researchers in computer science and/or engineering to intuitively understand the intrinsic structure and features of signalling pathways; and (ii) to propose a procedure to automatically determine flow speeds of different transductions leading intracellular responses to ultimate regulation, which are determined along with the transition speeds of Petri net.

Therefore, the simulation method on timed Petri net model of apoptosis may probably be an available one to provide a number of valuable results. As the future work, the following problems are open to be solved: (i) to simulate other Petri net modelled biological pathways; and (ii) to find efficient method for converting timed Petri net model to a continuous one that enables higher predictive precision.

In addition, the website so-called "Petri Net Pathways" (PNP for short) has been built up to open since 1st April, 2006. This website provides biological pathway models with Petri nets as well as corresponding detailed explanation by flash animation that can make the biologists intuitively understand intrinsic structure and features of biological pathways. Further, by employing the simulation techniques proposed in this paper, it is possible to mechanically simulate timed Petri net models and obtain valuable insights from the results of analyses and simulations. Now, PNP has presented three Petri net models of IL-1, G-protein and TPO signalling pathways. The rest Petri net models for the pathways such as IGF-1, IL-3, PDGF and TGF-beta signalling pathways are planned to be added soon.

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