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A mathematical model of the hsp70 regulation in the cell

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A mathematical model of the regulation process of the heat shock protein hsp70 in the cell is presented. The model describes the damaging effect of elevated temperature on proteins; the interaction of free hsp70 with injured proteins and its chaperone role in nascent protein translation; the relation between the amount of free hsp70 and the formation of the activated trimer form of the heat shock factor protein (HSF); the binding of activated HSF with the heat shock elements on the DNA; the transcription of mRNA of hsp70 and the synthesis of hsp70. The reaction of the model to a temporal rise in temperature shows an initial decline and a subsequent sharp rise to an ultimately increased level of free hsp70 in the cell. The response of the model to both a single and two consecutive heat shocks appears to closely resemble experimental data on hsp70 synthesis. This general agreement demonstrates the structure of the model to be sound and suitable as a basis for further modelling the complex tolerance mechanism of the cell.

Key words: Heat shock, heat shock protein, mathematical model.

1. Introduction

In recent years insight into the defence mechanisms of the cell to stress situations has increased rapidly. It has become apparent that heat shock proteins play an important role in the development of tolerance to heat and chemical stressors (Lindquist 1986, Subjeck and Shyy 1986, Morimoto *et al.* 1990, Li *et al.* 1991a,b, Maresca and Lindquist 1991, Nover 1991). While the hsp's normally present in the cell provide a certain degree of protection to proteins in the cell against the damaging effect of various stress conditions, it has been shown that a brief and moderate temperature increase can significantly raise the amount of hsp in the cell with a corresponding increase in protection.

The possibility of increasing the tolerance of cells to stress situations by a relatively small heat shock has created high expectations of the use of stress treatments in different fields of medicine and initiated much research into the mechanisms involved. However, while this research has revealed how many important parts of the complex mechanism of tolerance development function, an overall picture of the process has not emerged. One reason is that, in describing the observed behaviour of the components, it is often ignored that this behaviour could have been very different. The fact that the components—the proteins in the cell—behave as they do, originates in the system as a whole and consequently, to understand the behaviour of the parts one should study the whole. Or as Paul Weiss puts it:

> 'Although the brilliant progress of biochemistry keeps on increasing the list of opportunities for componental interactions, the rules of order which rigorously restrain them in such coordinated fashion as to yield a harmonious group performance of the collective can only be recognized, appreciated and properly described once we have raised our sights from the element to the collective system; and this means passing to a higher level of conceptualization' (Weiss 1972, page 8).

One way of gaining (quantitative) information about the 'collective system' is to describe all known parts and their interactions in a mathematical model. The behaviour of this model will then provide information about the complete system. To investigate the development of tolerance of cells to stress, two research groups—Molecular Cell Biology of the Utrecht University and Medical Physics of the University of Amsterdam—have developed a mathematical model of the regulation of the heat shock protein hsp70 as a first step in exploring the 'collective system'. The model is based on the assumption that the synthesis of hsp70 and its reaction to stress is a regulated and adaptive process. As has been discussed extensively in previous publications (Peper *et al.* 1987, 1988), the development of tolerance is a very complex adaptive mechanism because it incorporates the ability to adjust the process parameters to changing environmental conditions and to adapt to recurring short stimuli, which requires a series of interacting adaptive systems. Because of the immense complexity of the hsp70 regulation and the many interactions with other regulations, particularly of other members of the same heat shock family, the model developed is intended as a framework only, to be used as a basis for the development of more specific and accurate models.

1.1. *Biological basis of the model*

In many organisms the induction of heat shock proteins by moderate stress situations is accompanied by an induction of tolerance to more severe stresses (review: Lindquist 1986, Subjeck and Shyy 1986, Morimoto *et al.* 1990, Li *et al.* 1991a, Maresca and Lindquist 1991, Nover 1991). Although a few cases have been reported of tolerance induction without hsp synthesis (Hall 1983, Van Wijk and Boon-Niermeijer 1986, Boon-Niermeijer *et al.* 1986, 1988, Laszlo 1988, Aujame and Firko 1988), it seems clear that the increase in the synthesis of hsp's after a stress situation is the major protecting mechanism. A variety of early data indicate that particularly the proteins of the hsp70 family have a protective function against stress (Welch and Feramisco 1984, Riabowol *et al.* 1988, Li 1989, Bensaude *et al.* 1990, Li *et al.* 1991a,b). The development of the tolerance of the cell is only part of the complex task of hsp70. In the normal functioning of the cell, the family of hsp70 proteins plays an important role in processes of protein biogenesis such as protein synthesis and protein translocation (review: Freedman 1992, Georgopoulos and Welch 1993). The 70 kDa hsp is reported to associate with nascent polypeptides and other components of the translation machinery (Beckmann *et al.* 1990, Nelson *et al.* 1992, Frydman *et al.* 1994). Through interaction with hsp70 proteins the nascent proteins are supposed to be maintained in a stable conformation until their translation is completed.

In stress conditions, the structural integrity of proteins in the cell can be compromised, resulting in the exposure of otherwise hidden interactive domains. The binding of the hsp70 to these damaged proteins protects the interactive domains and

prevents the proteins from aggregating, providing the opportunity to refold. In the refolding reaction hsp70 probably function in concert with other chaperones.

Most of the present data about the role of molecular chaperones in protein folding have been obtained from *in vitro* assays using specific protein substrates. *In vitro*, the hsp70 family—in conjunction with other chaperones—have been shown to efficiently refold denatured substrate proteins (Freeman *et al.* 1995, Levy *et al.* 1995, Freeman and Morimoto 1996). The role of the members of the hsp70 family in this process has been observed to be partially different (Freeman and Morimoto 1996).

The binding of hsp70 to partially unfolded proteins after a stress condition depletes the pool of free hsp70 proteins and triggers the activation of genes encoding hsp's. Simultaneously, a preexisting transcriptional activator called heat-shock factor (HSF) is activated, promoting a rapid release of polymerase (Sorger *et al.* 1987, Wu *et al.* 1987). Among the many potential intracellular regulators of HSF, possibly the hsp's themselves—directly or indirectly—control the activity of HSF. Although several studies indicate a model in which the level of hsp's, particularly hsp70, negatively control the activity of HSF (Boorstein and Craig 1990, Baler *et al.* 1992, Abravaya *et al.* 1992, Mosser *et al.* 1993, Morimoto 1993), a more complex mechanism, as yet not clearly defined, may be involved. In addition, recent data indicate that an activation of HSF is not sufficient for the induction of hsp70 gene expression (Ovelgönne and Van Wijk 1995) and additional factors or the involvement of other HSE-binding factors may play a role (Yang *et al.* 1996). An interesting aspect is that the induction of hsp70 directly seems to influence the inducibility of hsp70. Changes in the thermo-sensitivity of the cell and the inducibility of hsp70 after a heat shock have been reported for the rodent CHO and BHK cells and the human HeLa and 293 cells (Mizzen and Welch 1988), the murine-SQ-1 and the human HCT-8 cells (Li and Mak 1989, the C3H10T1/2 mouse fibroblast cells (Tuijl *et al.* 1993) and the Reuber H35 hepatoma cell (Ovelgönne and Van Wijk 1995).

1.2. *The mathematical model*

The parameters of the mathematical model were derived from both published and unpublished data. Some parameters were derived from isolated measurements, others were determined from subprocesses in the regulated process. The exact value of the latter parameters was derived from the overall behaviour of the model. This possibility of approaching the parameters of the different subprocesses isolated from the total closed loop control process is the great power of the model.

The temporal changes in the induction of hsp70 synthesis of H35 rat hepatoma cells after a treatment with two consecutive heat shocks of 42°C were set as a guide for the dynamic behaviour of the model. Many of the complex and often as yet undefined processes indicated above had to be simplified for the model. The members of the hsp70 family found in mammalian cells interact with a variety of unfolded polypeptides in the cytosol, mitochondria and endoplasmic reticulum. The mammalian cytosolic hsp70 members, including the partially constitutive hsp70 (also named hsc70) and the highly inducible hsp68 (also named hsp70), have been shown to transiently associate with nascent polypeptides. All are generally assumed to be involved in the protection of the cell to stress (Freeman and Morimoto 1996) and this first model of the regulation of hsp70 combines the constitutive and inducible family members. The resulting model describes the binding of hsp70 to nascent

proteins and to damaged proteins after a stress condition. It describes how, after a heat shock, the binding of hsp70 to denatured proteins depletes the pool of free hsp70, which triggers the activation of genes encoding hsp's. The decrease in the level of free hsp70 results in a decrease in the influence which hsp70 normally exerts on the heat shock factor, allowing HSF to bind to the heat shock element in a transcription-activated form. The resulting increased hsp70 expression replenishes the pool of unbound hsp70's and represses HSF activity: the level of free hsp70 in the cell is determined by a regulation loop, the input of which is the binding of hsp70 to HSF. The different stages in this regulation are depicted in figure 1(a).

2. The biological basis of the model

Figure 1(a) shows a scheme of the biological functions of hsp70. The structure of the model describing these processes in mathematical form is shown in figure 1(b). The mathematical model consists of five main blocks, each describing one or more processes partaking in the functions of hsp70 and its synthesis. The connections between the blocks correspond to the protein interactions between the processes in the different blocks. The heavy printed connections indicate the main route for the regulation loop. The model is developed with the mathematical simulation program Simulink, an extension to the Matlab technical computing language. A short description of the functioning of Simulink is given in appendix B where the modelling of the mass balance—abundantly used in the model—is demonstrated.

The biological processes and the mathematical expressions describing them will be treated successively. Where necessary, an explanation of the simulation in Simulink is added.

2.1. *Block 1: the denaturation of proteins, its effect on protein synthesis and the binding of the hsp70 to denatured and nascent proteins*

Block 1 describes the denaturation of proteins upon an increase of temperature, the inhibition of protein synthesis during an excess of denatured proteins and the binding of hsp70 to denatured and nascent proteins. These processes determine the level of free hsp70, which is the main output of the block.

The binding of hsp70 to proteins can be observed in two experimental circumstances:

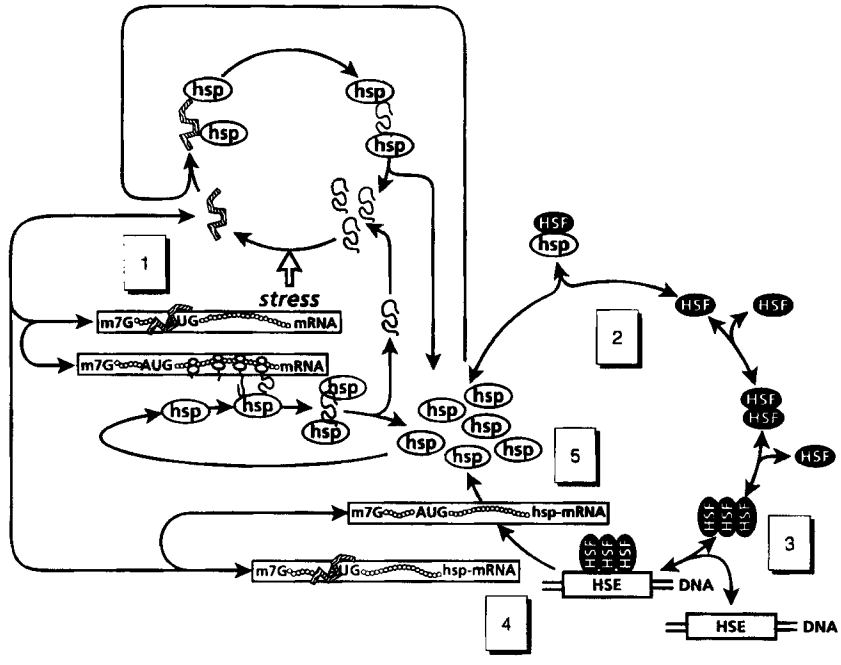
(1) During protein synthesis and translocation, when surfaces normally buried in the native structure of the proteins are transiently exposed and thus susceptible to off-pathway associations (Beckmann *et al.* 1990, Hartl *et al.* 1992, Hansen *et al.* 1994).

(2) During periods of stress, when properly folded and/or assembled proteins expose normally hidden, interactive, domains.

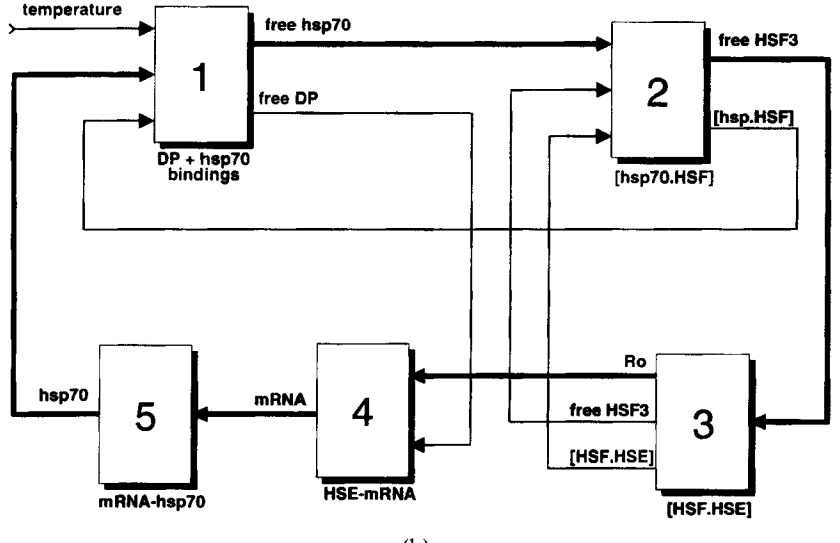
The amount of free hsp70 in the cell (hsp_f) as a function of time is obtained when from the total amount of hsp70 (hsp_t) its complexed forms with injured proteins (DP), heat shock factor (HSF) and nascent proteins are subtracted:

$$hsp_f(t) = hsp_t(t) - [hsp \cdot DP](t) - [hsp \cdot HSF](t) - [hsp \cdot Nascent](t) \qquad (1)$$

For brevity, in the following the index (t) is omitted.



(a)



(b)

Figure 1. A diagram of the biological funcitons of hsp70 (figure 1(a)) and the structure of the model describing the biological processes in mathematical form (figure 1(b)). The mathematical model is divided into five main blocks. The connections between the blocks show the protein interactions between the processes in the different blocks. The main route for the regulation loop is represented by heavy printed connections. The blocks are also shown in figure 1(a), to indicate the biological processes simulated in them: Block 1: the denaturation of proteins, its effect on protein synthesis and the binding of hsp70 to denatured and nascent proteins; Block 2: interaction of hsp70 with HSF; Block 3: interaction of HSF with HSE; Block 4: synthesis of hsp70mRNA; Block 5: synthesis of hsp70.

2.1.1. *Denaturation of proteins.* An increase of the temperature of the cell causes a change in the conformation of cellular proteins. The dynamics of this denaturation is complex. The rate of denaturation appears to differ across cellular proteins, depending as well on the period of hyperthermic treatment as on the temperature.

For the model, the denaturation of proteins during hyperthermic treatment has been qualified using differential scanning calorimetry with a steady increase of the temperature (Lepock *et al.* 1988). We used the fractional protein denaturation, obtained by this method, to procure our data, although this approach has a disadvantage in that it allows both endothermic and exothermic reactions to occur at normal and supranormal temperatures.

The fractional protein denaturation as a function of the temperature, V_{den}, in the range of 37–45°C, was calculated from data obtained from CHL V79 cells. The curve obtained was in the model approximated by:

$$V_{\text{den}} = \left(1 - \frac{0.4}{e^{T-37}}\right) \times 0.03 \times 1.4^{T-37} \tag{2}$$

T is the temperature in °C.

When V_{den} is multiplied by the amount of native proteins, P_{native}, the amount of protein that denatures per unit of time, Q_{den} results. P_{native} is obtained when from the total amount of native proteins susceptible to denaturation in the range of 37–45°C (P_0)—approximated for the model as 10% of the total amount of proteins in the cell—the denatured proteins (DP) are subtracted and the renatured proteins (P_{ren}) are added:

$$Q_{\text{den}} = V_{\text{den}} \cdot P_{\text{native}} = V_{\text{den}} \cdot (P_0 - DP + P_{\text{ren}}) \tag{3}$$

In the model the amount of denatured proteins which complex with hsp70 is determined by a mass balance. In contrast to the other mass balances in the model where there is an interaction between two substances to form a complex, in this mass balance free hsp70 interacts with both the denatured and the native proteins: free hsp70 (hsp_f) binds to free DP (DP_f), reducing the amount of free DP in the cell, while the released (renatured) proteins (P_{ren}) are added to the pool of native proteins in the cell. The complexation is described by the following relation:

$$[\text{hsp} \cdot \text{DP}] = \frac{1}{\tau}\int (k_{h+} \cdot \text{DP}_f \cdot \text{hsp}_f)\,\text{d}t - \frac{1}{\tau}\int (k_{h-} \cdot [\text{hsp} \cdot \text{DP}])\,\text{d}t \tag{4}$$

The first term describes the forming of the hsp.DP complexes while the last term describes the dissociation of the complexes into renatured proteins. The time constant τ is the integration constant of the integrator used. The amount of free denatured proteins, DP_f, is obtained when the amount of denaturing proteins per unit of time, Q_{den}, is integrated and the amount of DP's complexed with mRNA and hsp70 is subtracted:

$$\text{DP}_f = \frac{1}{\tau}\int \text{Q}_{\text{den}}\,\text{d}t - [\text{mRNA} \cdot \text{DP}] - \frac{1}{\tau}\int (k_{h+} \cdot \text{hsp}_f \cdot \text{DP}_f)\,\text{d}t \tag{5}$$

2.1.2. *Inhibition of protein synthesis.* At elevated temperatures the translation of most mRNA's is inhibited in mammalian cells, probably at the initiation step of the process (McCormick and Penman 1969, Hickey and Weber 1982, Duncan and Hershey 1984, 1989). This inhibition is, directly or indirectly, related to heat-shock

induced denaturation or modification of the eukaryotic protein synthesis initiation factors. A temperature increase can compromise the activity of various eukaryotic initiation factors, of which the two main targets seems to be eIF2 and eIF4E (Duncan and Hershey 1989, Duncan *et al.* 1995). However, the mechanisms that regulate this inhibition, especially at mild heat shock temperatures, are still poorly understood (Duncan and Hershey 1984). Studies from Matts *et al.* (1993) suggest that denatured proteins are a signal for this translational inhibition. In the model we have simulated the inhibition of protein synthesis by a binding of DP to mRNA in the cell.

The amount of mRNA complexing with denatured proteins is determined by a mass balance between the total amount of mRNA ($mRNA_t$) and free DP:

$$[\text{mRNA.DP}] = \frac{1}{\tau}\int (k_{m+} \cdot (\text{mRNA}_t - [\text{mRNA} \cdot \text{DP}]) \cdot \text{DP}_f)\, dt$$

$$- \frac{1}{\tau}\int (k_{m-} \cdot [\text{mRNA} \cdot \text{DP}])\, dt$$

The level of synthesis—R_{synt}—is calculated as the amount of mRNA not complexed with DP, divided by the total amount of mRNA in the cell:

$$R_{synt} = \frac{\text{mRNA}_t - [\text{mRNA} \cdot \text{DP}]}{\text{mRNA}_t} \tag{7}$$

R_{synt} should be unity in a normally functioning cell.

The amount of hsp70 available to bind to nascent peptide chains (Nascent) is directly related to R_{synt}. Nascent is determined by multiplying the maximum amount of nascent sites available by R_{synt}. The maximum amount of available sites for hsp70 is obained by multiplying the maximum amount of nascent chains by the average of the available sites for hsp70 per nascent chain (set at 4 in the model). The amount of nascent sites complexed with hsp70 is deterined by a mass balance between Nascent and free hsp70.

$$[\text{hsp} \cdot \text{nascent}] = \frac{1}{\tau}\int \left(k_{n+} \cdot \{(\text{Nascent} - [\text{hsp} \cdot \text{Nascent}]) \cdot \text{hsp}_f\}\right) dt$$

$$- \frac{1}{\tau}\int (k_{m-} \cdot [\text{hsp} \cdot \text{Nascent}])\, dt \tag{8}$$

Figure 2 illustrates the modelling of the different processes of this block in Simulink. The block consists of three interacting mass balances. While the simulation of the mass balances between respectively DP and mRNA and between hsp70 and Nascent follows the general description in the appendix, the simulation of the denaturation and renaturation of protein in the cell is more complex and will therefore be discussed in some detail below.

The relation between the temperature and the speed of denaturation, given in (2), is implemented in the formula block F1 of figure 2. Its output, V_{den}, is multiplied by P_{native} (multiplier M1) to obtain Q_{den}, the amount of protein that denatures per unit of time. As described in (3), $P_{native} = P_0 - DP + P_{ren}$. DP is obtained by integration of Q_{den}, while P_{ren} is the integral of the negative input of the mass balance 'binding hsp70 to DP', which is the dissociating amount of hsp · DP complex; the proteins renatured by their binding to hsp70. Therefore, $P_{ren} - DP$ is integrated with integrator 'native proteins', I_1. The initial value of I_1 is P_0, the total amount of native

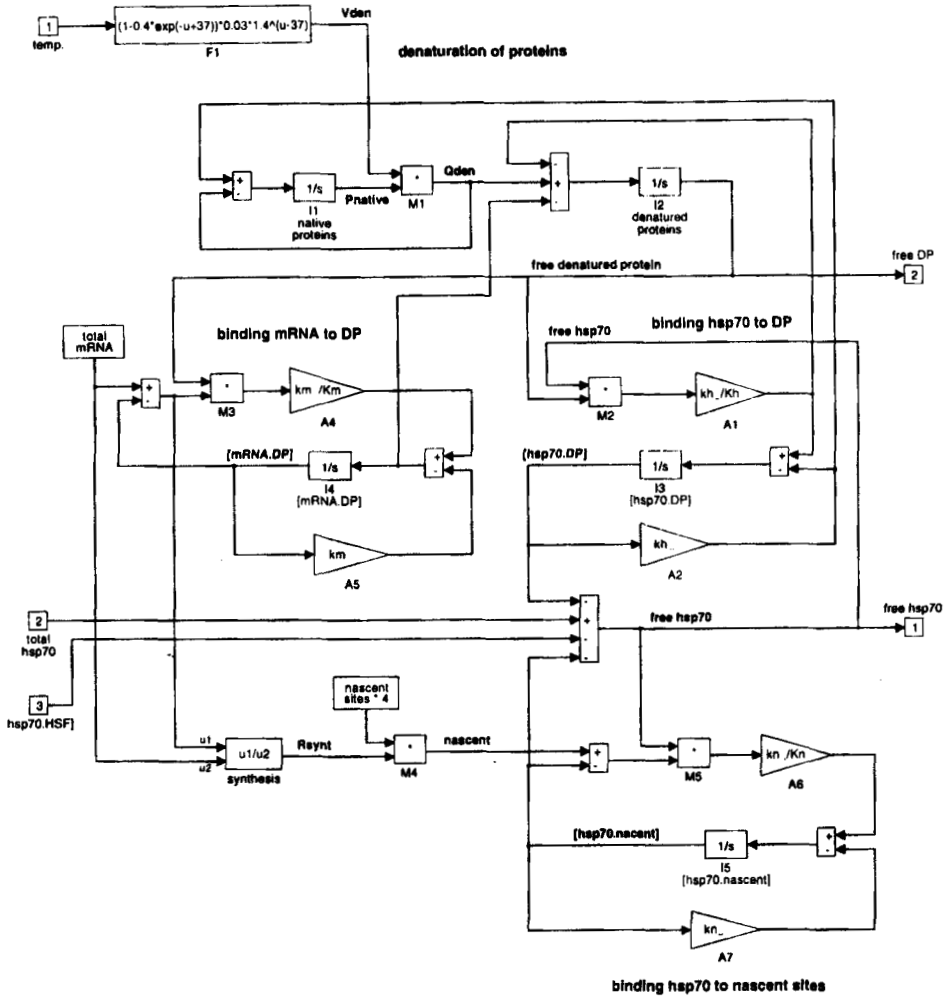


Figure 2. Block 1: 'DP and hsp70 bindings'.

protein in the cell susceptible to denaturation between 37 and 45°C, the current temperature range of the model.

The amount of free injured proteins, DP_f, is obtained when Q_{den} is integrated and the amount of DP bound to MRNA and hsp 70 is subtracted from the result. In the model the last two quantities are differentiated and subtracted from Q_{den} before integration. The differentiated [mRNA · DP] is found at the input of integrator I_4, while the positive ('charging') input of the mass balance 'binding of hsp70 to DP' provides the differentiated amount of DP complexed with hsp70.

The effect of a decrease in the level of synthesis on the binding of hsp70 to nascent sites is simulated by multiplying the number of available nascent sites by R_{synt}, which is computed in the function block 'synethesis' as described in (7). The output of this multiplication is the input of the mass balance with free hsp70 in the cell. The output is the amount of hsp70 bound to nascent chains. As described, this level will decrease after a heat shock when the synthesis goes down.

2.2. *Block 2: interaction of hsp70 with HSF*

Activation of heat shock genes only occurs when the trimer form of the heat shock factor (HSF) occupies a specific DNA sequence called the HSF binding element (HSE), which is located in or near a heat shock gene (Wu 1984, Xiao and Lis 1988, Wu *et al.* 1990, Morimoto *et al.* 1992). Under physiological conditions most of the HSF in the cell exists in an inactive state bound to hsp70 (Craig and Gross 1991, Abravaya *et al.* 1992, Baler *et al.* 1992, Beckmann *et al.* 1992, Mosser *et al.* 1993). Upon stress the hsp70-bound HSF is released, changes into a trimer form and migrates to the cell's nucleus, where it is assumed to be the determining factor in the activation of heat shock genes.

The model assumes that hsp70 only binds to the monomer form of HSF (HSF_1). The alternative, a binding of hsp70 to the monomer, the dimer (HSF_2) as well as the trimer (HSF_3) was also simulated but the outcome of the simulation did not appear perceptibly different and will not be discussed here. Free hsp70, then, is in a mass balance with free HSF_1.

$$[\text{hsp.HSF}_1] = \frac{1}{\tau}\int(k_{1+} \cdot \text{hsp} \cdot \text{HSF}_1)\,\mathrm{d}t + \frac{1}{\tau}\int(k_{1-} \cdot \text{hsp} \cdot \text{HSF}_1)\,\mathrm{d}t \tag{9}$$

In the model the trimer form of HSF is obtained from the monomer in two steps. First the dimer is made from the monomer with a mass balance in which both inputs are the monomer.

$$\text{HSF}_2 = \frac{1}{\tau}\int(k_{2+} \cdot \text{HSF}_1^2)\,\mathrm{d}t - \text{HSF}_3 - \frac{1}{\tau}\int(k_{2-} \cdot \text{HSF}_2)\,\mathrm{d}t \tag{10}$$

Secondly, from the dimer and the monomer the trimer is made with another mass balance:

$$\text{HSF}_3 = \frac{1}{\tau}\int(k_{3+} \cdot \text{HSF}_1 \cdot \text{HSF}_2)\,\mathrm{d}t - [\text{HSF}_3 \cdot \text{HSE}] - \frac{1}{\tau}\int(k_{3-} \cdot \text{HSF}_3)\,\mathrm{d}t \tag{11}$$

the amounts of HSF in dimer and trimer form and the amount of HSF_3 bound to HSE are subtracted from the total amount of HSF in the cell to obtain the level of free HSF_1. In this subtraction, HSF_2 has to be multiplied by two and HSF_3 by three to obtain the proper amount of HSF in relation to the monomer form.

$$\text{HSF}_1 = \text{HSF}_t - [\text{hsp} \cdot \text{HSF}_1] - 3\text{HSF}_3 - 3[\text{HSF}_3 \cdot \text{HSE}] - 2\text{HSF}_2 \tag{12}$$

The simulation of this block is given in figure 3.

2.3. *Block 3: interaction of HSF with HSE*

The interaction of trimeric HSF positioned on an HSE, activates arrested RNA polymerase II molecules to start the synthesis of hsp70-mRNA (Kingston 1991). However, the acquisition of the DNA-binding ability by trimerization of HSF does not always lead to an increased hsp sysnthesis (Mivechi *et al.* 1994, Ovelgönne and Van Wijk 1995, Ovelgönne *et al.* 1995). The model assumes that the concentration of free HSF_3 and its binding characteristics to HSE are the determinants of their complexation. These HSE binding characteristics are different among the promotors of the hsp70 family (Topol *et al.* 1985, Cohen and Meselson 1988, Xiao and Lis 1988) probably due to different configurations of these promoters. In the model they are represented by one binding constant. Their interaction is assumed to follow the law of mass action.

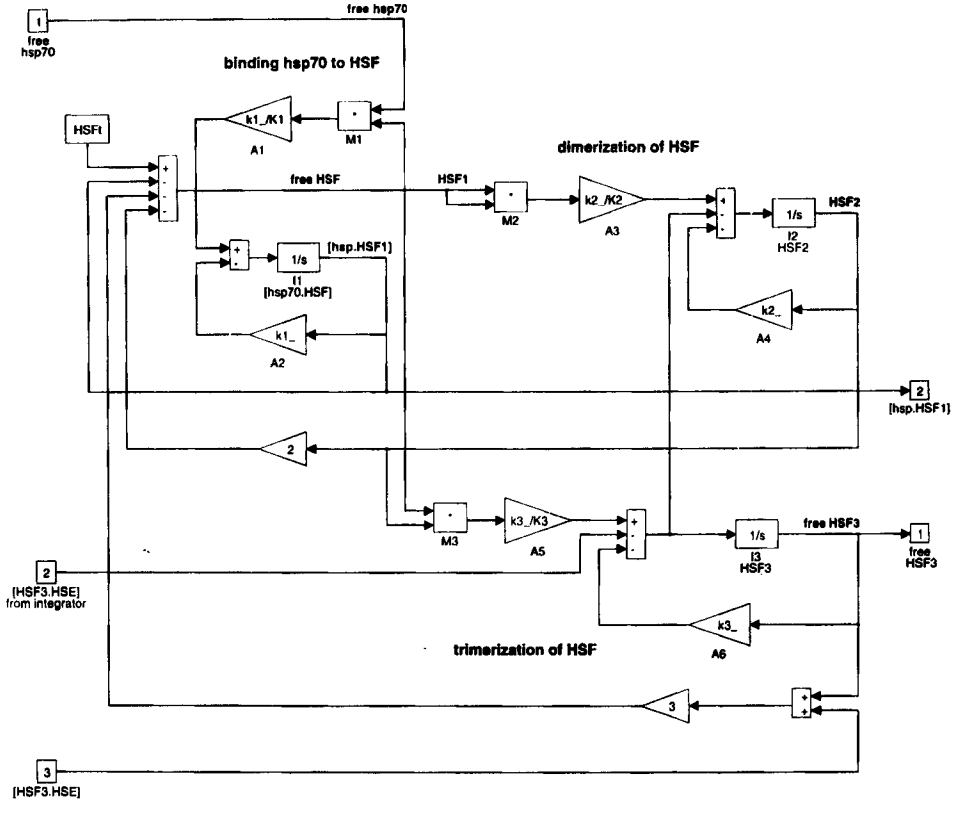


Figure 3. Block 2: '[hsp70 · HSF]'

It is assumed that the binding of HSF_3 to the HSE of hsp70 takes place to the same degree as the binding of HSF_3 to the total amount of HSE at the DNA and that, consequently, the level of synthesis of hsp70 is linearly related to the degree of occupation of HSE by HSF_3. In the HSF · HSE block the complexation of HSF_3 and HSE is simulated with a mass balance.

$$[HSF_3 \cdot HSE] = \frac{1}{\tau}\int (k_+ \cdot HSF_{3f} \cdot HSE_f)\,dt - \frac{1}{\tau}\int (k_- \cdot [HSF_3 \cdot HSE])\,dt \qquad (13)$$

The amount of unoccupied HSE is the total amount of HSE (HSE_t) minus the amount complexed with HSF:

$$HSF_f = HSE_t - [HSF_3 \cdot HSE] \qquad (14)$$

The ratio R_o of the level of complex HSF_3 · HSE and the level of the total amount of HSE in the cell determines the level of hsp70mRNA synthesis, simulated in the next block. R_o is computed in the function 'degree of HSE occupation' and is the output of the block:

$$R_o = \frac{[HSF_3 \cdot HSE]}{HSE_t} \qquad (15)$$

The simulation of this block is given in figure 4.

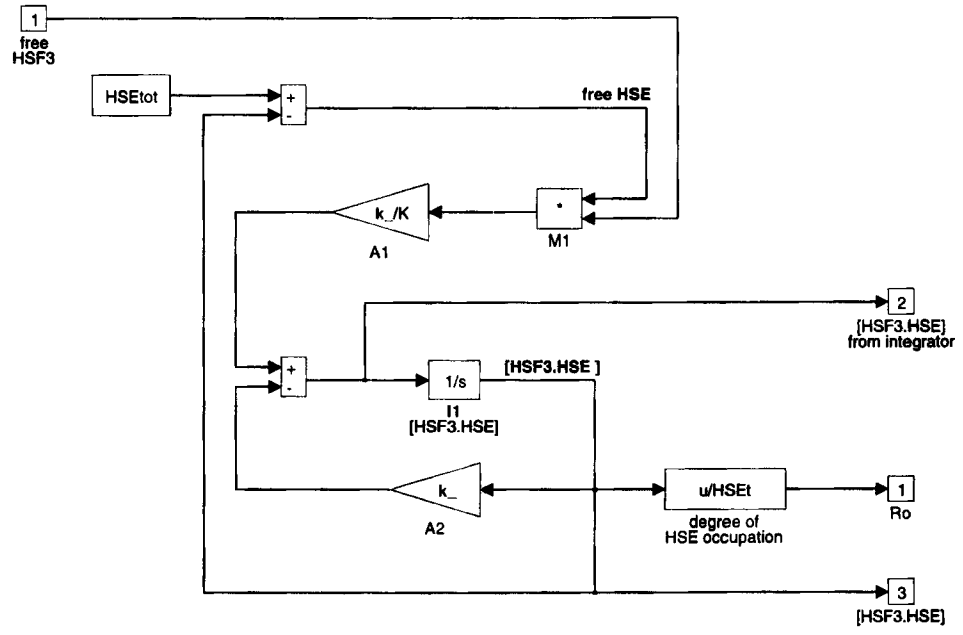


Figure 4. Block 3: '[HSF · HSE]'.

2.4. *Block 4: synthesis of hsp70mRNA*

During and after stress the translations of various mRNA's, including those of hsp's, decreases, influencing the level of the total protein synthesis in the cell. This effect simultaneously determines the level of hsp synthesis and the stability of mRNA, as inhibition of mRNA translation lowers the decay of this protein.

Block 4 describes the relation between the HSE occupied by transcriptional active HSF and the level of hsp70mRNA actively engaged in the synthesis of its protein. The hsp70 gene transcription rate is taken to be linearly related to the activation of HSE. The model incorporates two types of post-transcriptional processes, i.e. mRNA stability and translational control. With respect to hsp70mRNA it has been shown that this mRNA decays with a half-life of 1–2 h in unstressed cells (Theodorakis and Morimoto 1987), but that its stability increases during a heat shock or in the presence of translation inhibitors (DiDomenico *et al.* 1982a, b), Banerji *et al.* 1986).

The production of mRNA for hsp70 is calculated as $R_o \cdot V_{transcr} \cdot N_{gene}$, in which $V_{transcr}$ is the maximum transcription speed and N_{gene} the number of genes producing mRNA for hsp70, set at 4 in the model. A delay of 0.5 h is added to account for the transcription time.

$$\text{hsp70mRNA}_f = \frac{1}{\tau}\int (R_o \cdot N_{gene} \cdot V_{transcr}(t-0.5))\,dt - [\text{hsp70mRNA} \cdot \text{DP}] - \frac{1}{\tau}\int \left(\frac{1}{\tau_m} \cdot \text{hsp70mRNA}_f\right) dt \tag{16}$$

The produced hsp70-mRNA is integrated to obtain the total amount of hsp70mRNA in the cell. The integrator is also part of a mass balance between the mRNA and the free injured proteins in the cell, DP. To account for the half life time

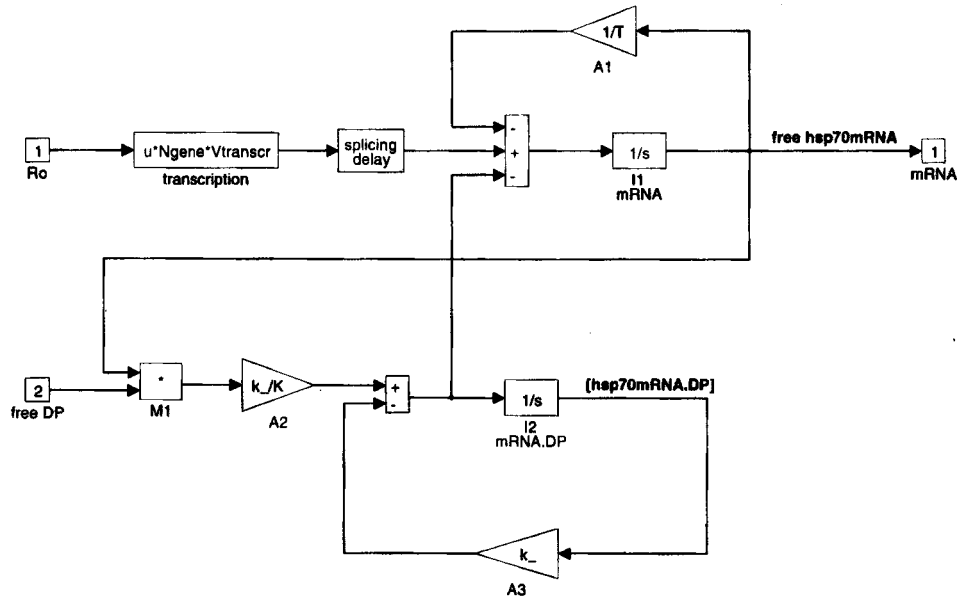


Figure 5. Block 4: 'HSE-mRNA'.

T_m of the mRNA molecules in the model, a fraction $1/T_m$ of the output of the integrator is subtracted from its output. In the model the half-life of translatable hsp70 mRNA is set at 2 h. The mRNA bound to DP in the mass balance is not affected by this feedback and consequently does not decay.

$$[\text{hsp70mRNA} \cdot \text{DP}] = \frac{1}{\tau}\int (k_+ \cdot \text{IP}_f \cdot \text{hsp70mRNA}_f)\,\mathrm{d}t - \frac{1}{\tau}\int (k_- \cdot [\text{hsp70mRNA} \cdot \text{DP}])\,\mathrm{d}t \tag{17}$$

The simulation of this block is given in figure 5.

2.5. *Block 5: synthesis of hsp70*

This block describes the translation of viable hsp70mRNA into its protein. The rate-limiting sub-process of translation is the initiation event. The process of protein elongation is thought to be temperature independent in the range of interest. Once synthesized, proteins are marked for destruction with a varying half-life. Hsp70 does function for several days (Landry *et al.* 1982, Li and Werb 1982).

The level of translatable mRNA is multiplied by the speed of elongation, V_{transl}, to obtain the production of hsp70 protein molecules in the cell. This result is integrated to obtain the level of hsp70 in the cell, hsp_t. A fraction of the level of hsp_t is fed back to the input of the integrator to account for the decay of hsp70, set at 36 h in the model:

$$\text{hsp}_t = \frac{1}{\tau}\int (\text{hsp70mRNA}_f \cdot \text{V}_{\text{transl}})\mathrm{d}t - \frac{1}{\tau}\int \left(\frac{1}{\tau_{\text{hsp}}} \cdot \text{hsp}_t\right)\mathrm{d}t \tag{18}$$

The simulation of this block is given in figure 6.

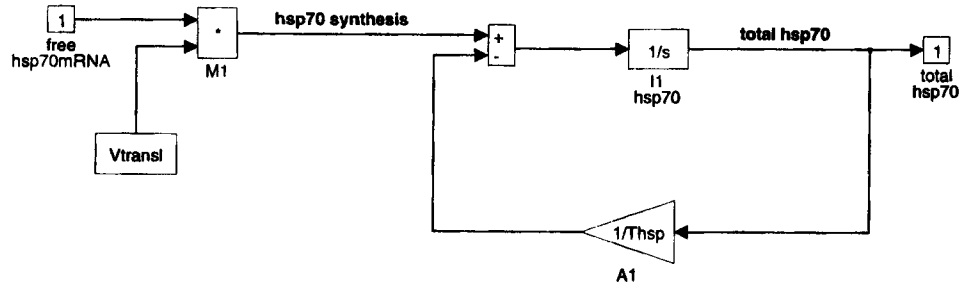


Figure 6. Block 5: 'mRNA-hsp70'.

3. Model performance

The model was developed to simulate the course of induction of hsp70 synthesis in Reuber H35 rat hepatoma cells after a heat shock. An incubation of H35 hepatoma cells at elevated temperatures has a marked effect on their rate of protein synthesis, the synthesis of members of the hsp70 family and the development of thermotolerance (Schamhart *et al.* 1984, Van Wijk *et al.* 1993, Ovelgönne and Van Wijk 1995). The recuperation of the protein synthesis of the cell after a heat treatment depends on the temperature and the duration of the heat treatment. After a heat shock of 0.5 h at 42°C it takes about 6 h for a total recovery to be reached.

The simulation of the heat-induced synthesis included the hsp70 family members hsp68 and hsp70. Although the synthesis of hsp68 is barely detectable in control cells while the synthesis of hsp70 is semi-constitutive, the kinetics of the increase and decrease of their synthesis are similar. To compare the outcome of model simulations with experimental findings, the data of both these members of the hsp70 family were combined in the model parameter values.

3.1. *Simulations*

For the development of the model all data available from the literature and unpublished data were used. When available, data from Reuber H35 cells were used. The parameter values used in the model were, when possible, determined at control and heat shock conditions from mammalian cells. Some kinetic constants in the model had to be determined from the model behaviour itself (see discussion). Table 1 shows the values of the parameters used in the simulation. The behaviour of the mathematical model was examined with respectively a single heat shock and two consecutive heat shocks. The outcome of these simulations was compared with experimental data from Reuber H35 hepatoma cells on the synthesis of the hsp70 family members. Figure 7 shows a simulation made with the model of the effect of a half-hour heat shock of 42°C upon the level of the relevant substances in the hsp70 regulation. The figure shows from top to bottom and from left to right: (1) the temperature; (2) the effect of the heat shock on the level of denatured protein. In the simulations, the level of denatured protein at 37°C is 4×10^{-7} M; (3) the effect of the heat shock on the protein synthesis in the cell; (4) this figure is the most important result of the modelling. It shows the effect of the heat shock on the level of free hsp70 in the cell, an effect which up to now cannot be measured *in vivo*. The figure shows a fast increase in the level of hsp70 after the heat shock. Before this increase there is a period when the level of free hsp70 is strongly decreased due to its binding

Table 1. The parameter values used in the simulations.

DP + hsp70 bindings block		
K_h	10^{-8}	M
k_{h-}	0.2	h^{-1}
K_m	2×10^{-6}	M
k_{m-}	10	h^{-1}
K_n	10^{-10}	M
k_{n-}	100	h^{-1}
NC	3.5×10^{-6}	M
hsp70 · HSF block		
K_1	2×10^{-8}	M
k_{1-}	100	h^{-1}
K_2	4×10^{-10}	M
k_{2-}	100	h^{-1}
K_3	4×10^{-10}	M
k_{3-}	100	h^{-1}
HSF_t	4×10^{-8}	M
HSF · HSE block		
K	10^{-9}	M
k	100	h^{-1}
HSE_t	5×10^{-9}	M
HSE-mRNA block		
$V_{transcr}$	1.2	s^{-1}
N_{gene}	4	
T	2	h
K	2×10^{-6}	M
k	10	h^{-1}
mRNA-hsp70 block		
V_{transl}	0.1	s^{-1}
T_{hsp}	24	h

to denatured protein; (5) the decrease of the level in free hsp70 causes a strong increase in the level of HSF not bound to hsp70. The figure shows the amount of HSF trimer. The levels of the other forms of HSF, the monomer and the dimer, are not shown. (6) The complexation of HSF_3 to HSE shows a flattening at the top when most HSE is bound. (7) The level of free mRNA—not bound to injured proteins—shows a strong decrease during the time the level of IP is high. (8) The synthesis of hsp70 initially goes down after the heat shock followed by a strong increase (9). This figure shows the increase in the level of hsp70 in the cell after the heat shock and the decrease in the level of hsp70 bound to nascent chains. Apparently, the hsp70 that is released in this way directly binds to IP, as it has no visible effect on the level of free hsp70.

Figure 8 shows the effect of two successive heat shocks of 42°C. The second heat shock is applied 16 h after the first one, at a moment when the level of free hsp70 has increased strongly after the first heat shock. The figure shows that the effect of the

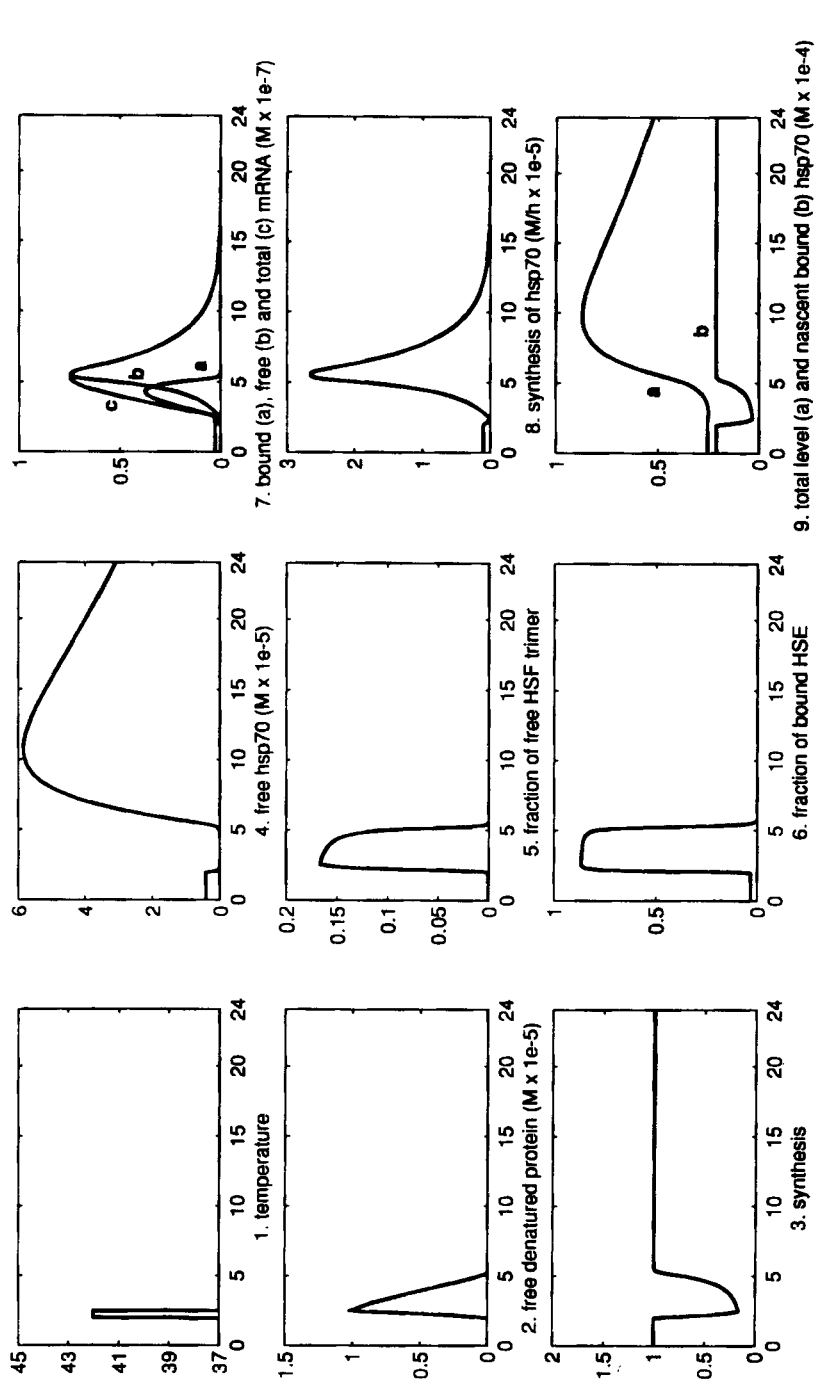


Figure 7. Simulation of the effect of a $\frac{1}{2}$ h heat shock of 42°C upon the level of the relevant substances in the hsp70 regulation: (1) Temperature in °C. (2) Level of denatured protein in M. (3) Protein synthesis relative to normal synthesis. (4) Free hsp70 in M. (5) HSF trimer as fraction of total (monomer) HSF. (6) Fraction of bound HSE. (7) Bound, free and total mRNA in M. (8) Synthesis of hsp70 in M. (9) Total and nascent bound hsp 70 in M.

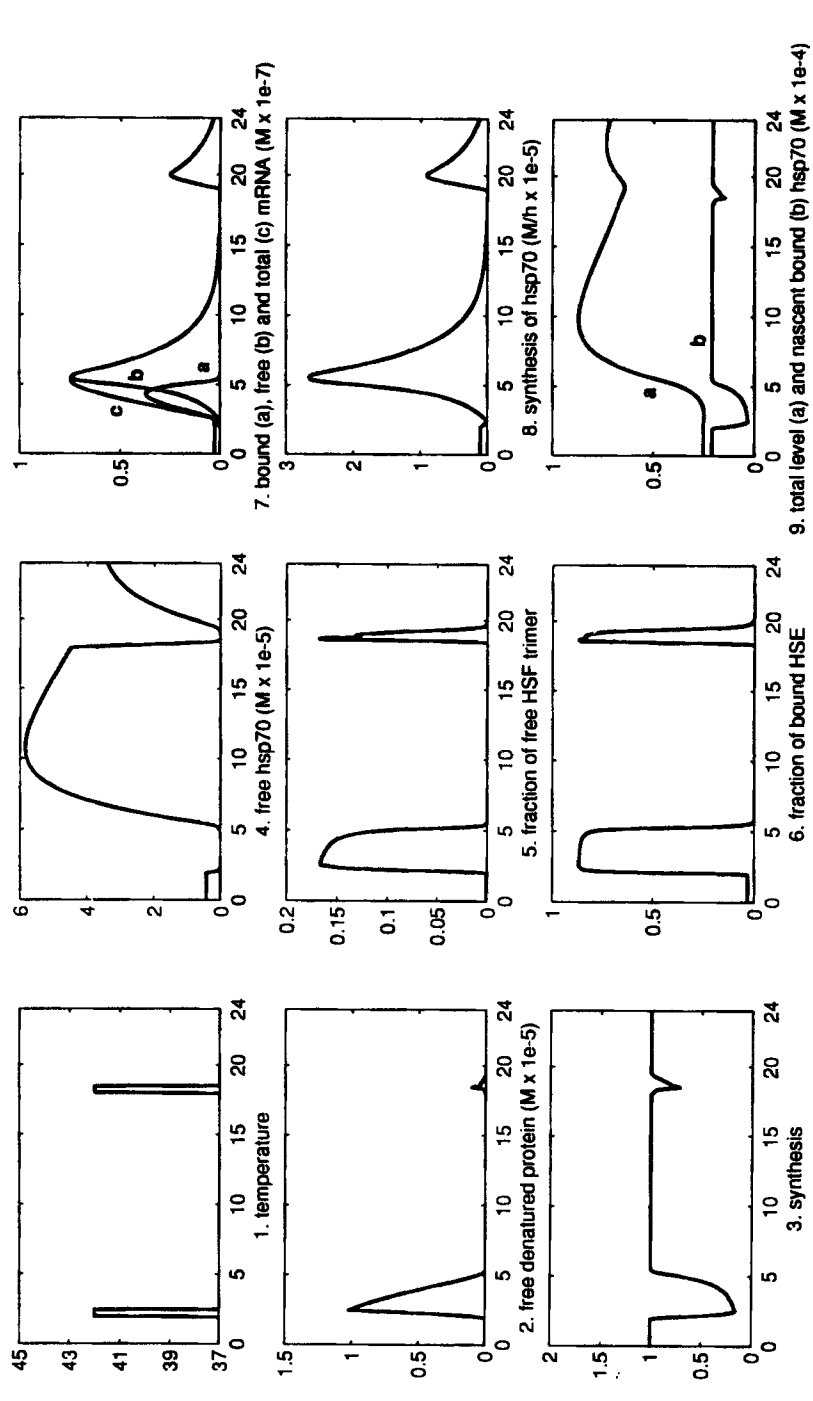


Figure 8. The effect of two successive heat shocks of 42°C. The second heat shock is applied 156 h after the first one, at a moment when the level of free hsp70 has increased strongly after the first heat shock. The effect of the second heat shock is substantially less than the effect of the first one, apparently due to the large increase in the level of free hsp70 in the cell.

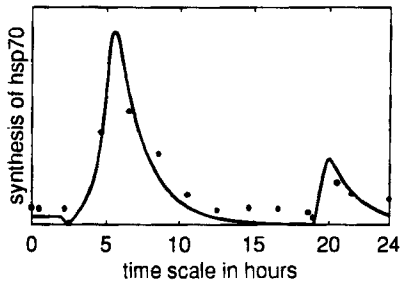


Figure 9. Measurement results of the synthesis of hsp70 in Reuber H35 cells after a heat shock of 0.5 h 42°C at 2 h (dots). A comparison of these experimental data with the computed curve from figure 8 (solid curve) shows that the state of tolerance induced by a heat shock of 0.5 h 42°C to a large extent resembles the data obtained with a model simulation under the same conditions.

second heat shock is substantially less than the effect of the first one, apparently due to the large increase of the level of free hsp70 in the cell.

3.2. *The effect of an enhanced temperature on the synthesis of hsp70 in Reuber H35 rat hepatoma cells*

Figure 9 compares experimental data of the synthesis of hsp70 after a heat shock of 42°C in Reuber H35 cells (dotted curve) with a simulation by the model. The hsp synthesis is maximal at 4 h after the heat shock at 2 h and is at basal level again at 10 h after the heat shock. When 16 h after the first heat shock a second similar heat shock was given, the synthesis of the hsp70 family was severely depressed compared with the first induction. A comparison of the experimental data with the computed curve from figure 8 (solid curve) shows that the state of tolerance as induced by 0.5 h 42°C largely resembles the data obtained with the mathematical model.

4. Discussion

The regulation process can be divided into two different functioning loops: the regulation of the constitutive level of hsp70 and the reaction of the regulation to a stress situation. This division originates in the highly non-linear characteristics of the trimerization process, which allows small disturbances to be regulated with a fairly small amplification, while large disturbances are amplified to such a high extent that 'saturation' of the regulation loop takes place.

The regulation of the constitutive level of free hsp70—in the model assumed to be the input of the loop—and of the levels of the other processes in the loop depends on the rate constants of the mass balances used and on the constants: decay time of hsp70mRNA and hsp70, speed of translation and transcription and number of genes for hsp70. The rate constants for the mass balances between free hsp70 and HSF and between HSF and HSE were chosen to obtain the estimated constitutive levels of HSF and HSE. Together with the other constants, they produce an open loop gain for small disturbances of about six, which is in the range one may expect for a biological process.

In the model, the chosen rate constant for the binding of hsp70 to HSF yields a constitutive level of free hsp70 of about 20% of the total concentration of hsp70. Recently, a reversible conformational change in hsp70 at higher temperatures

(>41°C) *in vitro* has been reported (Leung *et al.* 1996), which in the model might be translated into a change in rate constant for the binding of hsp70 to HSF. A change in this rate constant directly influences the constitutive level of free hsp70. However, the exact value of this rate constant is not very important because the constitutive level of free hsp70 *in vivo* is not known and its effect on the kinetics of the loop is not large, as will be discussed. The affinity of the monomer form of HSF to the dimer and trimer form was taken to be higher than that of HSF to hsp70 to ensure that most of the free HSF transforms into the activated trimer.

After a heat shock, the level of free hsp70 goes down when hsp70 binds to denatured proteins. Because of the nonlinear behaviour of the trimerization process of HSF and the small time constant of this process (Abravaya *et al.* 1992, Morimoto *et al.* 1992, Ovelgönne and Van Wijk 1995), a decrease in the level of free hsp70 almost immediately results in a total activation of the available HSF in the cell. This in turn causes an immediate complete binding of the activated HSF to all HSE on the DNA and a proportionate increase of the synthesis of mRNA of hsp70. In the model, the effect a heat shock has on the synthesis of hsp70 is therefore mainly determined by the time the level of free hsp70 remains low after stress and by the transfer of the loop (its input–output relation) when all HSE is activated. The constants: decay time of hsp70mRNA and hsp70, speed of translation and transcription and number of genes for hsp70 determine the transfer of the loop in this situation. The product of these constants determines their effect on the synthesis and because they are chosen somewhere in the range of probability—most available data about their value is very unspecific—the total effect was chosen to obtain the synthesis to be in the expected range. When, after a heat shock, most of the free hsp70 available is bound to denatured protein and the amount of free hsp70 has decreased to a very low level, the synthesis of mRNA of hsp70 increases rapidly. The induced synthesis consequently depends on the level of free hsp70 before the heat shock, and the amount of denatured proteins produced by the heat shock. For the first heat shock, the level of free hsp70 is the constitutive level. By the time the second heat shock is applied the level of free hsp70 has increased considerably. This implies that much of the DP induced by a second heat shock will be bound by the large amount of available free hsp70. Only when most of the free hsp70 is bound to DP, will the synthesis of mRNA of hsp70 be induced. After its initial rise following the first heat shock the level of free hsp70 declines steadily and it should be clear that it is not only the level of the first heat shock—and the resulting increase in the amount of free hsp70—which determines the effect of a second heat shock, but also the moment when the latter is applied.

During the time when free hsp70 is low, almost all HSE is activated and changes in the (low) level of free hsp70 have no effect on the level of activated HSE. The consequence is that the loop does not regulate. This implies that the process at that moment follows its own course, which is determined by the constants mentioned above. The time during which the level of free hsp70 remains low after a heat shock depends directly on the time there are free denatured proteins in the cell. Although the speed of denaturation of the protein in the cell was determined as a function of the temperature of the heat shock—as discussed in the description of block 1—there is no data available about the amount of free denatured protein as a function of time after a heat shock. The processes which determine the level of free denatured proteins were distinguished to be: the total amount of denatured proteins, the amount of free hsp70, the ability of hsp70 to bind to denatured proteins, the time hsp70 remains

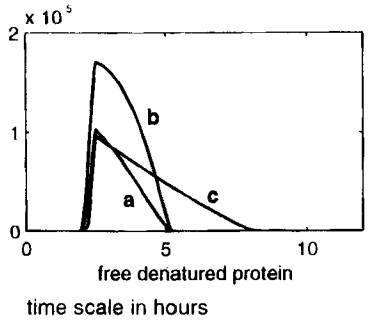


Figure 10. Simulations of the ways in which the cell can fight denatured proteins; the area under the curve determines the chance of survival for the cell: (a) The amount of denatured proteins as simulated in the model (figure 7). (b) The amount of denatured proteins when the synthesis of new hsp70 does not decrease after a heat shock and the binding of hsp70 to nascent proteins remains complete. (c) The effect when the synthesis after a heat shock becomes zero and remains that low as long as denatured proteins are present.

bound to denatured proteins, the amount of hsp70 bound to nascent proteins and the decrease in synthesis after a heat shock.

In the first instance after a heat shock, the amount of hsp70 able to bind to denatured proteins is determined by the constitutive level of free hsp70. However, when the synthesis of proteins in the cell goes down after the heat shock, the hsp70 bound to nascent proteins is released adding substantially to the pool of hsp70 able to bind to denatured proteins. In fact, from the functioning of the model it became clear that in the first phase of the renaturing process of denatured proteins, the amount of hsp70 becoming available from nascent proteins plays a major role. This indicates that for the behaviour of the model the way the regulation of the synthesis is simulated is an important factor. However, not much is known about how and why the synthesis decreases. In the model the amount of free denatured proteins have been linked to the degree of synthesis by binding the denatured proteins to the mRNA in the cell. Although the resulting behaviour of the synthesis after a heat shock is very similar to the measurements on the H35 cell (Van Wijk *et al.* 1993, Ovelgönne *et al.* 1995) when appropriate values for the rate constant and time constant of this process are used, this construction leaves some important questions.

After a stress situation the main concern for the cell is to fight the danger by binding as many hsp70 proteins as possible to denatured proteins. For the cell there appear to be two ways to achieve this goal. One way is to use all available hsp70 to this end which means that the activity of the protein synthesizing machinery has to be reduced to free the hsp70 bound to nascent proteins. The other way is to increase the synthesis of new hsp70 as fast as possible to increase the pool of available hsp70. In the model the former is assumed. Figure 10 simulates both ways. Figure 10(a) shows the amount of denatured proteins simulated in figure 7. Figure 10(b) shows the amount of denatured proteins when protein synthesis, including the synthesis of new hsp70, does not decrease after a heat shock and the binding of hsp70 to the nascent proteins remains complete. The effect is that the amount of free denatured proteins about doubles.

Possibly the model has to be extended to allow for selective initiation of hsp70-mRNA molecules. Such a preferential translation is well known for Drosophila cells but not for mammalian cells (DiDomenico *et al.* 1982b).

In addition, figure 10(c) shows the effect when the synthesis after a heat shock becomes zero and remains that low as long as denatured proteins are present. The time taken to renature all denatured proteins now depends on the amount of hsp70 available at the time of the heat shock and consequently is much longer. For the cell the area under the curve determines the chance of survival and it is clear that the way the model functions at present provides the smallest area and consequently the highest chance of survival among the possibilities shown. Apparently, for the cell, this compromise between using in the first instance all available hsp70 and a moderate delay of the increase in the synthesis of new hsp70 is the best way of tackling the danger represented by denatured proteins.

It remains questionable whether, in the model, the level of synthesis should be allowed to depend on the level of free denatured proteins when this level itself, via the binding of denatured proteins to nascent bound hsp70, depends on the degree of synthesis. The consequence of this construction became visible in the model as it tends to counteract the difference between the effect of heat shocks of different temperature.

An alternative process, namely a direct influence of temperature upon synthesis, is a possibility which has to be investigated.

The results of the simulations with the model appear to closely resemble the behaviour of the tolerance process of the cell as is known from the literature and from the authors own measurements, demonstrating that the assumptions underlying the model are sufficiently sound. This is important because it shows that, notwithstanding the enormous complexity of the process, its overall time-dependent behaviour can be represented in mathematical form. An important goal of this first attempt of modelling the tolerance mechanism of the cell was to develop a basic structure on which future developments can be built. The priority now is to expand the model to include the different heat shock proteins belonging to the hsp70 family. The present model incorporates the behaviour of hsp68 as well as hsp70. The kinetics of the two hsp's differ in that hsp70 shows a much larger constitutive level than hsp68 while the reaction to a heat shock is much more pronounced in hsp68 than in hsp70. The simplification in the model in this respect might explain the difference in base line level between the first and the second heat shock in the measured and computed curves in figure 9.

Another step will be to adapt the model to the effects of long lasting changes in temperature compared with the short heat shock applied in the present simulations and to define the positive and negative adapting components of the tolerance process. These regulatory components become essential after long lasting changes in environmental conditions—discussed extensively in a previous publication (Peper *et al.* 1987: figure 7)—and can be expected also to play an important role in the tolerance process described in the present paper. The behaviour of cells during and after long lasting temperature changes has been studied in a previous paper (Van Rijn *et al.* 1995). H35 hepatoma cells were adapted to proliferate under normally lethal hyperthermic stress conditions. The cells showed a rapid decay of the thermoresistance and a decrease in heat shock protein synthesis when returned to 37°C.

The mechanisms underlying the reactions of the cell to different stimuli will also be investigated. Research done by our group has demonstrated large differences in the reaction of cells to different forms of stress. This implies that the tolerance mechanism contains memory allowing different stimuli to be recognized and that every form of disturbance excites its own defence mechanism. This is in accordance with the theories set forth in previous publications (Peper *et al.* 1987, 1988). However, not much is known about the way these different mechanisms function and interact in the cell and some basic questions have to be answered first. For instance, the question whether the tolerance response of a cell to stress is a learning of the processes involved to deal with the effect of a new stress condition or whether it is the expression of existing tolerance of an already tolerant cell. In the model presented the latter is assumed, but further evaluation is necessary.

A great difficulty in the development of the model was the interpretation of measurement results. The often detailed reports published in recent years on the molecular interactions involved in the regulation of hsp70 and the role of hsp70 in the adaptation to environmental changes show highly deviating results. The mathematical model described in this paper is based on the assumption that the synthesis of the hsp70 in the cell is a regulated process. Measurements in a regulated loop are extremely difficult to interpret due to the interrelation of the different parts of the process, which tends to counteract the effect of disturbances, while the feedback itself masks the dynamics of the individual processes. Especially in the present process, where many interacting branches determine the level of functioning of the regulation components, it is very difficult to interpret the outcome of experiments. This also demonstrates the importance of developing a model: it provides the possibility to establish the relation between the inputs and outputs of parts of the process and to study the functioning of the interactions between its components. With the model a better insight has been gained into how changes in the functioning of the different components on the hsp70 auto-regulated process depend not only on changes of environmental conditions but also, and even more, on the characteristics of the regulation loop itself: its complexity, the stability of its components, the stability of the loop gain and the presence of time delays and non-linearities.

An additional value of the model is that it can be used to predict the behaviour of the process: a change in the process parameters in the model will affect its behaviour in more or less the same way as a change of the corresponding parameters of the process *in vivo* will affect the behaviour of the biological process. This purpose directed interaction between the mathematical model and the biological reality allows for a better attuning to problems concerning the process *in vivo* than would be possible without the model.

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Appendix

The simulation program

The model is developed with the mathematical simulation program Simulink. Simulink is an extension to the Matlab technical computing language. In its use, Simulink closely resembles the old analogue computer, its main building blocks being amplifiers, integrators, multipliers and summing blocks. The extensive Simulink library also contains signal generators, oscilloscopes and a multitude of linear and non-linear function blocks. In addition, the user can define any self developed function block for future use while any part of the simulation circuit can be merged into a new block with a menu for the parameters used. Any block can be changed or duplicated without limitation. Through its modular structure, Simulink allows for the simulation of very complex systems and an easy and fast adaptation of the model parameters to the outcome of new measurements.

In figure A1 some much used function blocks are shown:

- Amplifiers.
- Integrators, indicated as 1/s, s being the complex Laplace operator. The integrator can be given an initial value.
- Summing blocks; the number of inputs to be summed or subtracted can be chosen.
- Constants.
- Multipliers; the number of signals to be multiplied can be chosen.
- Function blocks for the application of Matlab functions.
- Input and output ports for blocks.

The application of the Simulink program will be easily understood by anyone familiar with analogue modelling. In this appendix the modelling of the interaction of two substances in a liquid following the law of mass action is demonstrated. The

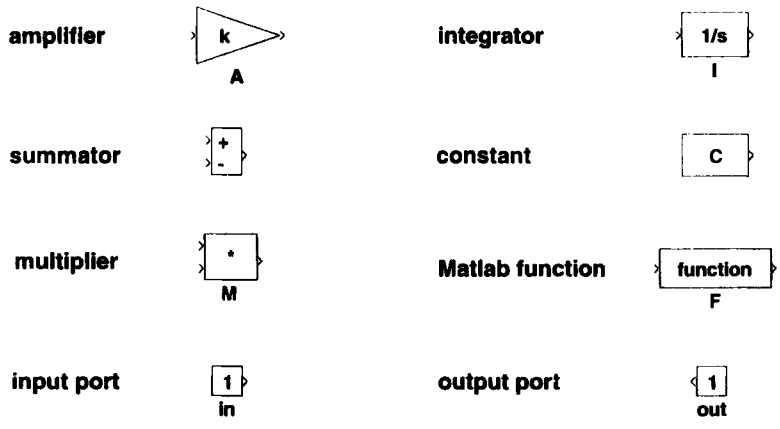

Figure A1. Some much used function blocks in Simulink.

different forms which the mass balance—abundantly used in the model to describe the interactions of the proteins in the cell—can have are treated to elucidate the way Simulink works. However, for those new in the field of modelling and simulation a study of the literature on the subject (Chestnut and Mayer 1951, Truxal 1995) is advisable.

The mass balance

A reaction between two substances A of concentration A and B of concentration B results in their complexed form C of concentration C. This can be symbolized by:

$$\mathrm{A} + \mathrm{B} \underset{k_-}{\overset{k_+}{\rightleftarrows}} \mathrm{C} \tag{A1}$$

k_+ and k_- are the reaction constants determining the velocity of the reaction in both directions and the level of A, B and C in equilibrium.

The reaction can be described with three differential equations (Rubinow and Segel 1980):

$$\frac{\mathrm{d}A}{\mathrm{d}t} = -k_+ \cdot A \cdot B + k_- \cdot C \tag{A2}$$

$$\frac{\mathrm{d}B}{\mathrm{d}t} = -k_+ \cdot A \cdot B + k_- \cdot C \tag{A3}$$

$$\frac{\mathrm{d}C}{\mathrm{d}t} = k_+ \cdot A \cdot B - k_- \cdot C \tag{A4}$$

Because differentiating in computer simulations is often problematic, these equations are integrated:

$$A = A_0 - \frac{1}{\tau}\int k_+ \cdot A \cdot B\,\mathrm{d}t + \frac{1}{\tau}\int k_- \cdot C\,\mathrm{d}t \tag{A5}$$

$$B = B_0 - \frac{1}{\tau}\int k_+ \cdot A \cdot B\,\mathrm{d}t + \frac{1}{\tau}\int k_- \cdot C\,\mathrm{d}t \tag{A6}$$

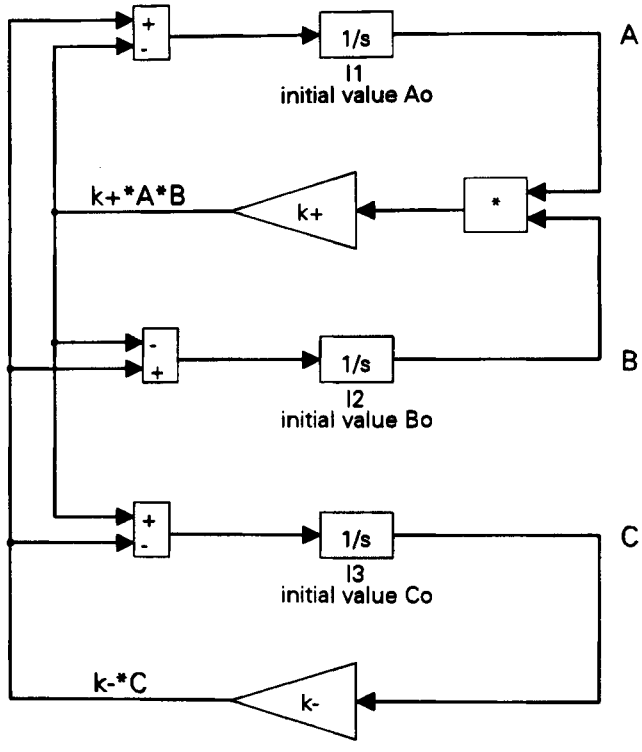

Figure A2. Simulation of the mass balance.

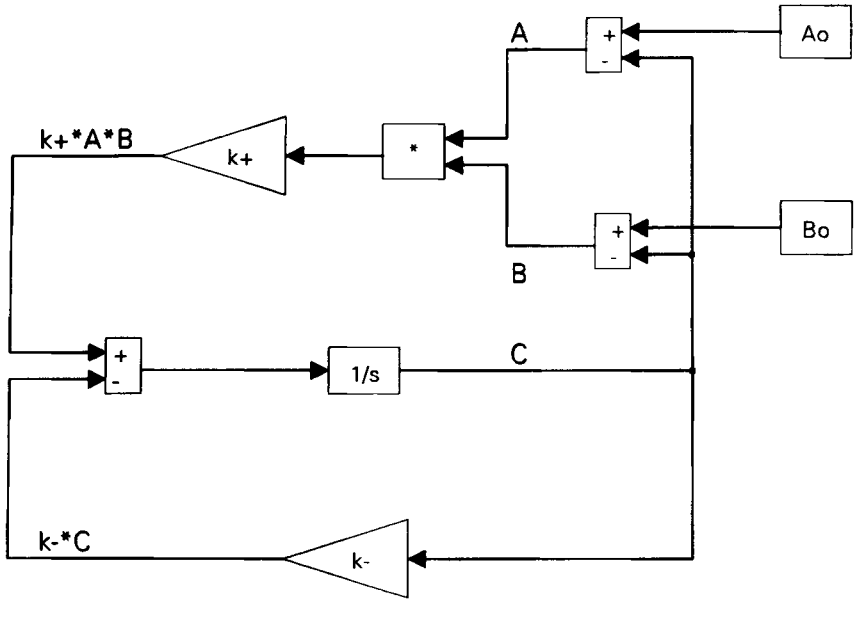

Figure A3. An alternative way of simulating the mass balance.

$$C = C_0 + \frac{1}{\tau}\int k_+ \cdot A \cdot B\,dt - \frac{1}{\tau}k_- \cdot C\,dt \tag{A7}$$

In equilibrium the differential equations become:

$$C = \frac{k_+}{k_-}A \cdot B \text{ or } C = \frac{A \cdot B}{K} \text{ in which } K = \frac{k_-}{k_+} \tag{A8}$$

In many cases k_- and k_+ are not known, only their quotient: the equilibrium constant of the reaction K, and the velocity of the reaction *in vivo*. As can be seen from the differential equations, the velocity of the process at a certain moment not only depends on k_- and k_+ but also on the values of A, B and C at that moment and consequently is not a constant. In the model k_- is chosen to obtain the desired reaction velocity in the particular situation of the simulated process with respect to the different levels of the interacting protein and the rate constant K; then k_+ follows from (A8): $K = k_-/k_+$.

The mass balance is simulated in figure A2. To explain the simulation of the three differential equations, it is easiest to start with the last one, equation (A7). To obtain C, $k_+ \times A \times B - k_- \times C$ has to be integrated. The first term is obtained by amplifying the product of A and B by k_+, the last term by amplifying C with k_-. The last value is then subtracted from the first one to obtain the input for integrator I_3. A and B are obtained in the same way, except that $k_+ \times A \times B$ is subtracted from $k_- \times C$ to obtain the input for the integrators I_1 and I_2. In fact, the input for the integrators I_1 and I_2 and for integrator I_3 is equal but has an opposite sign: any increase in the output of I_3 is accompanied by an equal decrease in the outputs of I_1 and I_2. This of course is what happens in reality: the forming of a certain concentration of the complex C is accompanied by an equal decrease in the levels of A and B. In the model the input of I_3 is in some cases directly subtracted from the input of I_1 or I_2.

The initial values of the integrators are A_0, B_0 and C_0. When equilibrium is reached, the inputs of the integrators become zero and consequently $k_+ \times A \times B = k_- \times C$, which agrees with (A8).

The model, the mass balances are assumed to start with the substances A_0 and B_0, after which the complex C is formed. Consequently $C_0 = 0$. Substitution of (A7) into (A5) and (A6) then shows that at any moment $A = A_0 - C$ and $B = B_0 - C$. This provides another way of simulating the reaction, as is shown in figure A3. In the model this way of simulating the mass balance is used in most cases, but the simulation of figure A2 and the mixed form are also used.