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On the kinetics of peptide binding to MHC proteins

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Abstract

An experimental study of kinetics of peptide binding to MHC proteins [S. Witt, H. McConnell, Acc. Chem. Res. 26 (1993) 442 (and references therein)] showed an unusual phenomenon of the so-called 'negative' $t_{1/2}$ plots (where $t_{1/2}$ is half time of reaching equilibrium concentration of peptide–protein complexes), which is the shorter $t_{1/2}$ at lower added peptide concentrations. The bell-shaped curve for $t_{1/2}$ as a function of peptide concentration is a seemingly peculiar effect, because in general, binding reactions go faster with an increase of reagent concentrations. It is shown that the suggested explanation of this phenomenon [S. Witt, H. McConnell, Acc. Chem. Res. 26 (1993) 442 (and references therein)] is misleading (and the numerical simulation used to support this explanation is inconsistent with the experimental data), so that the existence of 'negative' $t_{1/2}$ is in no way to be considered as an experimental indication of the two-step reaction of peptide binding to protein. This article gives a consistent explanation of the bell-shaped $t_{1/2}$ plots for peptide–protein association and obtains the criteria for its existence, based exclusively on the formal chemical kinetics analysis of the accepted peptide–protein binding model and briefly discusses the experimental data, which really confirm the existence of the two-step mechanism of binding. The analysis of the maximum location of the half-time curves indicates a controversy between the prediction of the two-step binding model and the experimental data [S. Witt, H. McConnell, Acc. Chem. Res. 26 (1993) 442 (and references therein)]: either more complicated mechanism is involved in peptide–MHC binding or experimental data [S. Witt, H. McConnell, Acc. Chem. Res. 26 (1993) 442 (and references therein)] are not quite accurate.

Keywords: Two-step binding kinetics; Peptide–protein binding; MHC protein

1. Introduction

Peptide binding to MHC (major histocompatibility complex) proteins was studied intensively as it appears to be a central step in immune recognition [1,2]. In this article, we are interested only in a description of peptide–protein binding kinetics based exclusively on the methods of formal chemical kinetics and do not touch any specificity of proteins or peptides.

Witt and McConnell [1] performed an intensive experimental study of the kinetics of peptide–protein binding in a wide range of peptide concentration. They observed an interesting phenomenon, called 'negative' $t_{1/2}$ plots (half time $t_{1/2}$ is defined as the time required to reach half of the equilibrium concentration of MHC–peptide complexes), where $t_{1/2}$

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is a shorter at lower added peptide concentrations. It was found that $t_{1/2}$ was an increasing function of peptide concentration (the reaction gets slower) even for peptide concentrations that were much greater than the receptor (MHC molecule) concentration. This result seems rather unexpected, because an increase of the reagent concentration generally leads to the acceleration of binding reaction. When the peptide concentration reached a certain value, $t_{1/2}$ started to decrease (reactions got faster). Thus, the $t_{1/2}$ plot as a function of peptide concentration goes through a maximum (bell-shaped). The authors of Ref. [1] tried to explain the observed results on the increase of $t_{1/2}$ only at the region of low peptide concentration (less than that of protein). Unfortunately, their explanation is misleading, it contradicts the basic principles of chemical kinetics, and does not explain the bell-shaped plots for $t_{1/2}$, which is discussed further in detail.

According to Witt and McConnell [1], 'negative' $t_{1/2}$ plot indicates the existence of a two-step binding reaction. The first (induction) step is the release of an endogenous peptide, which occupies the MHC binding groove, and the second step is the binding of the added peptide to the 'empty' molecule. As will be shown here, the bell-shaped $t_{1/2}$ plots could be observed if peptide binding to protein were just a simple one-step binding reaction, and thus, it cannot be considered as experimental proof of the two-step peptide–protein complex formation. Then the question arises—what kind of experimental data would indicate the existence of the induction step in peptide–protein association. We briefly consider the kinetic data which definitely confirm the existence of two-step peptide–protein binding. We also discuss the controversy between the experimental data of Witt and McConnell [1] and the prediction of the two-step peptide–MHC binding model on the location of the maximum of the bell-shaped half-time curve.

2. Kinetics of peptide binding to MHC protein

In this article, we analyze the following model which was accepted by Witt and McConnell [1] to describe the binding kinetics of peptide to the MHC protein

$$\mathrm{M-P_e} \underset{k_e^-}{\overset{k_e}{\rightleftarrows}} \mathrm{M + P_e} \tag{1}$$

$$\mathrm{M + P} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \mathrm{C} \tag{2}$$

with initial concentrations: $[\mathrm{P}] = P_0$, $[\mathrm{M-P_e}] = M_0$ and $[\mathrm{C}] = 0$ at $t = 0$. Here M is the MHC protein, $\mathrm{P_e}$ is the endogenous peptide, P is the peptide which binds to the protein M, C is the notation for the peptide–protein complex M–P, and t is time. Reaction rate constants can be obtained from experimental data on the kinetics of binding, their theoretical calculation is very complicated [3–6] and requires rather detailed information about the system, which is often not available.

The authors of the paper [1] affirm that 'negative' $t_{1/2}$ plot appears to be a result of a slow reaction in Eq. (1) and a very fast reaction in Eq. (2), which goes to completion. As a result, for the higher concentrations of peptide, we need to wait longer to get more protein released through reaction in Eq. (1) to bind half of the added peptide. That is why $t_{1/2}$ increases with the increase of peptide concentration. They consider that 'negative' $t_{1/2}$ plots indicate the existence of the induction step in Eq. (1), because, as they believe, such dependence of $t_{1/2}$ on the added peptide concentration is not expected for a simple binding reaction in Eq. (2). This explanation is very controversial, because for low peptide concentrations reaction in Eq. (2) is the limiting step of formation of the complexes M–P (the rate of the formation of complexes is proportional to [P]), so that binding can not be controlled by the induction step in Eq. (1)). Moreover, this strange explanation of the effect considers only the region where 'the concentration of added peptide is less than that of the protein' [1] and does not cover the range of experimental data. Let us follow the logic of the authors of Ref. [1]. Then $t_{1/2}$ increases with the increase of peptide concentration, because reaction in Eq. (2) becomes faster and faster, while reaction in Eq. (1) is the limiting step of binding, and therefore, it takes longer time to get more protein released through reaction in Eq. (1) to bind the increasing quantities of the added peptide. The half time reaches the asymptotic value of $\ln 2/k_e$ (which is the half time of endogenous peptide dissociation) at peptide concentration slightly greater than that of protein and will not decrease with the

increase of peptide concentration. That contradicts the bell-shaped plots for $t_{1/2}$ observed in experiment. Thus, the authors [1] did not give an explanation of their experimental result on $t_{1/2}$ increase for peptide concentration higher than the protein concentration and for the bell shape of $t_{1/2}$ plots.

We will further show that the 'negative' $t_{1/2}$ plot appears to be a feature of the accepted kinetic model of the system without any artificial assumptions about the rates of reactions in Eqs. (1) and (2). Let us consider the whole range of peptide concentrations $0 < [P] < \infty$. From a general basis of chemical kinetics at low peptide concentrations ($[P] \to 0$, $[P] \ll M_0$) reaction in Eq. (2) will be the limiting step of the complex formation, and the reaction between protein and peptide can be described as simple binding M + P = M − P (especially when equilibrium dissociation constant of Eq. (1), $K = k_e/k_e - \gg M_0$). We will show later that this can be correct even for relatively high peptide concentrations, depending on the values of the kinetic constants.

So we will first consider a simple binding reaction

$$M + P \underset{k_2}{\overset{k_1}{\rightleftarrows}} C \qquad (3)$$

with initial conditions $[P] = P_0$, $[M] = M_0$ and $[C] = 0$ at $t = 0$.

Kinetic equation $d[C]/dt = k_1[M][P] - k_2[C]$ can be solved analytically in this case

$$[C(t)] = \delta_1\delta_2\{1 - \exp[-k_1(\delta_1 - \delta_2)t]\} / \{\delta_1 - \delta_2\exp[-k_1(\delta_1 - \delta_2)t]\} \qquad (4)$$

where

$$\delta_{1,2} = \left\{k_2 + k_1(M_0 + P_0) \pm \left[\{k_2 + k_1(M_0 + P_0)\}^2 - 4k_1^2 M_0 P_0\right]^{1/2}\right\}/(2k_1)$$

In the equation above, δ_1 corresponds to the sign '+' and δ_2, to the sign '−'. Using the definition of $t_{1/2}$ $[C(t_{1/2})] = C[(t \to \infty]/2$, we easily obtain from Eq. (4) the equation for the half time of complex formation for a simple binding reaction

$$t_{1/2} = \ln(2 - \delta_2/\delta_1)/[k_1(\delta_1 - \delta_2)] \qquad (5)$$

For high initial concentrations of peptide ($P_0 \gg M_0$) this equation gives a well known result $t_{1/2} = \ln 2/(k_1P_0 + k_2)$. Two examples for $t_{1/2}$ as a function of added peptide concentration are presented on Fig. 1. As seen on Fig. 1, Eq. (5) for $t_{1/2}$ as a function of initial peptide concentration is a curve with a maximum at peptide concentration approximately equal the concentration of protein M_0.

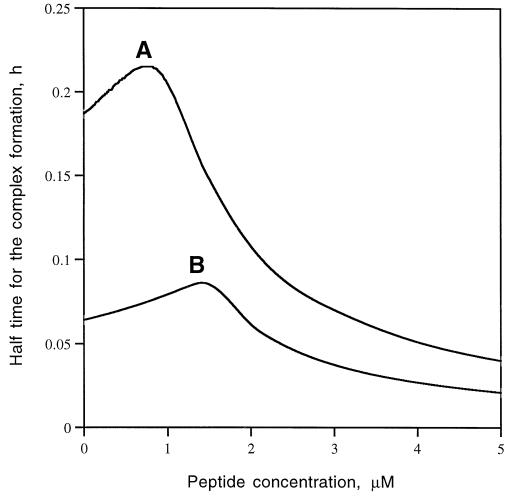

Fig. 1. Half time for the complex formation for the simple binding reaction M + P = C. (A) $M_0 = 1$ μM, $k_1 = 1000$ M^{-1} s^{-1} and $k_2 = 3 \cdot 10^{-5}$ s^{-1}. (B) $M_0 = 1.5$ μM, $k_1 = 10$ M^{-1} s^{-1} and $k_2 = 0.278 \cdot 10^{-7}$ s^{-1} ($t_{1/2}$ in case B is divided by 200 to bring the plots to the same scale).

In general, for a simple binding reaction, the half-time curve as a function of peptide concentration may have a maximum, or may not have it. It depends on only one parameter, which is the ratio of dissociation constant $K_d = k_2/k_1$ to the initial peptide concentration M_0, $r = K_d/M_0$. Really, we can rewrite Eq. (5) in dimensionless form

$$t^*_{1/2} = t_{1/2}k_1M_0 = \ln(2 - \delta_2/\delta_1) / \left[(1 + r + P_0/M_0)^2 - 4P_0/M_0\right]^{1/2} \qquad (6)$$

In Eq. (6), $t^*_{1/2}$ is the function of the two variables $r = K_d/M_0$ and P_0/M_0. The graph of $t^*_{1/2}$ for various values of the parameter r is shown on Fig. 2. Calculating the derivative $\partial t_{1/2}/\partial P_0$ from Eq. (5) at $P_0 = 0$, we can figure out that it changes the sign at $r = r_c = 1 - 1/(2 \ln 2) = 0.2787$, and $\partial t_{1/2}/\partial P_0 > 0$ when $r < r_c$. Thus, for a simple binding reaction in Eq. (3), the peak on the half-time curve exists only when

$$K_d/M_0 < r_c = 1 - 1/(2\ln 2) \qquad (7)$$

For peptides with higher affinity to protein (smaller value of the dissociation constant K_d), the peak on the $t_{1/2}$ curve (Fig. 2) is stronger and it occurs at peptide concentration approximately equal to that of protein. As the affinity diminishes and K_d/M_0 approaches the critical value r_c in Eq. (7), the $t_{1/2}$ peak gradually disappears, while its position shifts to a smaller value of P_0/M_0. It is interesting that the existence of a peak on a half-time curve depends on the value of the equilibrium dissociation constant $K_d = k_2/k_1$, but not on the kinetic rate constants k_1 and k_2 separately.

The authors of Ref. [1] overlooked a possible analytical way to figure out the existence of the bell-shaped half-time curve for a simple binding reaction and failed to discover it through numerical simulation, which is actually redundant once the direct analytical solutions in Eqs. (4) and (5) can be obtained. Thus, the 'negative' $t_{1/2}$ at very low peptide concentrations for peptide with relatively high affinity to protein is just an indication of a simple binding reaction, which is expected to describe the binding kinetics of the reactions in Eqs. (1) and (2) at low peptide concentrations. If peptide binding to protein was just a simple one-step reaction in Eq. (3), we could get a bell-shaped curve for $t_{1/2}$ as a function of peptide concentration, the left side of which is the 'negative' $t_{1/2}$ slope. Therefore, 'negative' $t_{1/2}$ phenomena does not occur only as a result of the existence of the induction step in Eq. (1), as authors of Ref. [1] affirm.

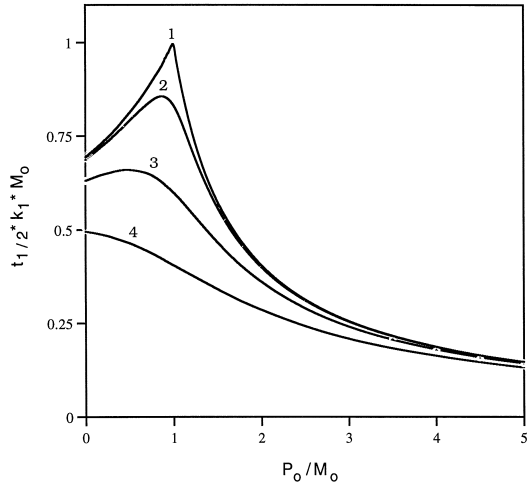

Fig. 2. Half-time curve (Eq. (6)) for a simple binding reaction (Eq. (3)) M + P = C, P_0 and M_0 are the initial peptide and protein concentrations, respectively. $1 - K_d/M_0 = 0$; $2 - K_d/M_0 = 0.01$; $3 - K_d/M_0 = 0.1$; $4 - K_d/M_0 = 0.4$.

The limiting value of $t_{1/2}$ for the simple binding reaction in Eq. (3) with initial conditions $[M] = M_0$, $[P] = P_0$ and $[C] = 0$ at $t = 0$, when peptide concentration goes to zero is $t_{1/2} = \ln 2/(k_1 M_0 + k_2)$. This can be easily understood, as for very low concentrations of peptide the protein is considered to be in an excess ($M_0 \gg P_0$) and then kinetics is described by a well known equation $[C(t)] = P_0\{1 - \exp[-(k_1 M_0 + k_2)t]\}$. Only when peptide concentration is zero (no peptide), then $t_{1/2}$ is not defined, but $t_{1/2}$ does not gradually tend to infinity with $P_0 \to 0$.

Nevertheless, there are two arguments against a simple binding reaction as a model for peptide reaction with MHC proteins. The first one is that for a simple binding reaction in Eq. (3), $t_{1/2}$ gets very small (approaches zero) with the increase of peptide concentration, which is opposite of what was found in the experiments of Witt and McConnell [1]. The asymptotic nonzero value of $t_{1/2}$, as shown further, is definitely an indication of the existence of the induction step in Eq. (1) in peptide–MHC binding. The second argument concerns the location of the maximum of the half-time curve. According to a simple binding reaction, the maximum of the curve occurs at peptide concentration which is less or approximately equal to the concentration of protein, while the experimental data [1] give the position of the maximum at peptide concentration much greater (about 400 times) than that of protein. This obvious controversy is discussed further in detail.

There is still a question then—why $t_{1/2}$ goes through maximum for the more complicated kinetic scheme given by reactions in Eqs. (1) and (2). When peptide concentration is getting higher, both reactions in Eqs. (1) and (2) are significant for kinetics of binding.

Let us consider the kinetics of two reactions in Eqs. (1) and (2) for a high peptide concentration limit (when peptide is in an excess), so that $P_0 \gg M_0$ and also assume $k_e^- = 0$, because the reverse reaction in Eq. (1) is suppressed by an excess of added peptide in this case. The initial conditions for these reactions (which correspond to experimental studies) are $[P] = P_0$, $[MP_e] = M_0$ and $[C] = 0$ at $t = 0$. Pep-

tide concentration can be considered constant in this case (because $P_0 - [P(t)] \leq (M_0 \ll P_0)$ and then we obtain

$$[C(t)] = C_{eq}\{1 + [k_e \exp[-(k_1 P_0 + k_2)t] - (k_1 P_0 + k_2)\exp(-k_e t)]/(k_1 P_0 + k_2 - k_e)\} \quad (8)$$

where $C_{eq} = M_0 k_1 P_0/(k_1 P_0 + k_2) = M_0/(1 + K_d/P_0)$, $K_d = k_2/k_1$.

For relatively low concentrations of peptide, when $k_1 P_0 + k_2 \ll k_e$ (but still $P_0 \gg M_0$), from Eq. (8), we get the following equation for complex formation $[C(t)] = C_{eq}\{1 - \exp[-(k_1 P_0 + k_2)t]\}$, which formally corresponds to the reaction of direct binding (Eq. (3)) at $P_0 \gg M_0$ without preceding Eq. (1). In this case, the time scale of the reaction in Eq. (1) is much shorter than that of the reaction in Eq. (2), which effectively leads to simple one-step binding kinetics in Eq. (3). At a high peptide concentration limit, reaction in Eq. (1) becomes the limiting stage of binding, while reaction in Eq. (2) is very fast, so that from Eq. (8) follows $[C(t)] = C_{eq}[1 - \exp(-k_e t)]$ and we get the asymptotic value of $t_{1/2} = \ln 2/k_e$ for high peptide concentrations. When there is still an excess of peptide, but its concentration decreases, $t_{1/2}$ increases as follows from Eq. (8) and is shown on Fig. 3. This result was confirmed in experimental studies of association kinetics at high peptide concentrations. These experimental data were obtained for the binding of HA 307–319 peptide (PKYVKQNTLKLAT) at high concentrations ($P_0 =$ 50 nM, 80 nM, 120 nM, 300 nM, 500 nM, 800 nM, 5 mm and 20 μM) to DR1DW1 molecules (at 3–5 nM concentration), which is one of the type of MHC protein (to be published).

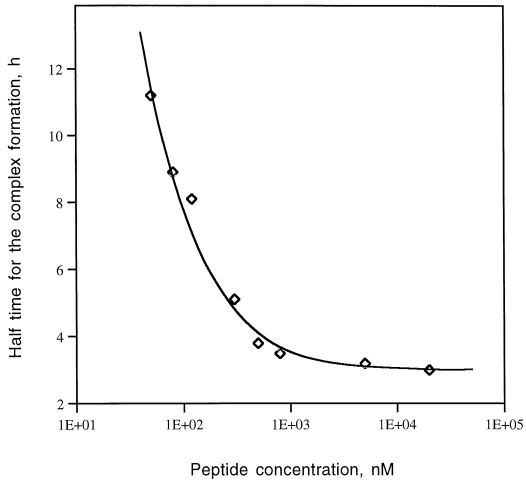

Fig. 3. Half-time plot for the formation of protein–peptide complexes, ◇ = experimental data, solid line represents analytical calculation using Eq. (8) with $k_e = 0.23\ h^{-1}$, $k_1 = 2\ \mu M^{-1}\ h^{-1}$ and $k_2 = 0.003\ h^{-1}$.

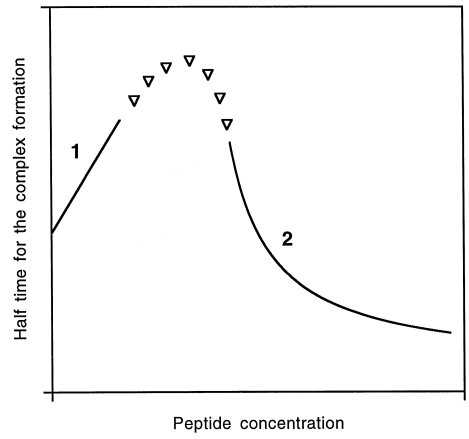

Fig. 4. 'Negative' $t_{1/2}$ plot for the kinetic mechanism given by Eqs. (1) and (2). (1) low peptide concentrations, (2) high peptide concentrations, ▽ = intermediate range of concentrations.

Once the limiting kinetics at very low and very high concentrations of peptide is understood, the kinetics at intermediate range of concentrations can be predicted and there is no need for numerical simulations in this case. To understand the whole picture of $t_{1/2}$ dependence on concentration, it was necessary to explain the possible increase of $t_{1/2}$ at low peptide concentrations (line 1 on the Fig. 4) and its decrease for high peptide concentrations (line 2 on the Fig. 4). Once we have pieces 1 and 2 of the curve, we can expect the whole curve to have a bell-shape form as shown as on Fig. 4. The more detailed picture, of course, depends on the value of the kinetic constants, so that the peak on the graphic is more or less noticeable. In case of the peptide with relatively low affinity to protein (or low initial protein concentration) so that $K_d/M_0 > r_c$ in Eq. (7), there will be no 'negative' $t_{1/2}$ in the low peptide concentration region (region of simple binding). In this case, we may expect the half-time curve to

exhibit 'regular' behavior, i.e., it will be decreasing gradually to the asymptotic value of $\ln 2/k_e$ with the increase of the initial peptide concentration.

The maximum of half-time curve, according to the experimental data of Witt and McConnell [1] occurs at peptide concentration much greater (approximately 400 times) than the concentration of protein. That might seem to be an indication that simple binding is not a relevant description of peptide–MHC reaction, because it yields a noticeable maximum of half-time curve at peptide concentration approximately equal to that of protein. But this is not correct. The analysis of kinetic model given by Eqs. (1) and (2) allows us to evaluate the position of the maximum of the half-time plots. Really, let us consider again the region of high peptide concentrations $P_0 \gg M_0$, where the kinetics of binding is given by Eq. (8). Using this equation to determine half-time value $[C(t_{1/2})] = C_{eq}/2$ and calculating $\partial t_{1/2}/\partial P_0$ we get

$$\partial t_{1/2}/\partial P_0 = k_1 t_{1/2}(k_1 P_0 + k_2)^{-1} F(z), \qquad (9)$$

where $F(z) = (\exp(z) - 1)^{-1} - 1/z$, $z = (k_1 P_0 + k_2 - k_e)t_{1/2}$.

It can be checked (analytically or numerically) that the function $F(z) < 0$ for any z (and therefore, P_0). It means that when we have an excess of peptide $\partial t_{1/2}/\partial P_0 < 0$ and $t_{1/2}$ decreases with the increase of the initial concentration of peptide. Roughly, when $P_0 \geq 10 M_0$, we are already considering the descending side (line 2) of the half-time curve on Fig. 4. In the same manner, when there is an excess of protein $P_0 \ll M_0$, the model system described by Eqs. (1) and (2) tends to a simple binding reaction, the kinetics of which is given by Eq. (4). Then, for $P_0 \ll M_0$ we are on the ascending side (line 1) of half-time curve on Fig. 4. Therefore, the maximum of the half-time curve for the reactions in Eqs. (1) and (2) may be approached at peptide concentration of the same order of magnitude as protein concentration, or possibly at peptide concentration much lower than that of protein, depending on the values of reaction rate constants. The maximum on $t_{1/2}$ curve at peptide concentration approximately equal to that of protein was observed experimentally for DR2 MHC protein (I. Astafieva, Anergen, personal communication). Even if the maximum on $t_{1/2}$ curve is relatively small (the height of the maximum is not substantially greater than $\ln 2/k_e$), $t_{1/2}$ values should stop to increase when peptide concentrations are of the same order of magnitude as protein concentrations.

Thus, we come to an important conclusion that the maximum on the half-time graph should be located at peptide concentration less or of the same order of magnitude as protein concentration, but not at peptide concentration much greater than that of protein. In this respect, the prediction of a two-step peptide–MHC binding mechanism contradicts to the experimental data of Witt and McConnell [1]. The same contradiction occurred in the numerical simulations [1] of the two-step binding kinetics (see Appendix A). It means that either more complicated mechanism is involved in peptide–MHC binding, or that these experimental data are not quite accurate. Because of that, the only reliable proof of the existence of an induction step (Eq. (1)) is a nonzero asymptotic value of $t_{1/2}$ for high peptide concentrations. In Eq. (9), $F(z) \to 0$ when $P_0 \to \infty$ and therefore, $\partial t_{1/2}/\partial P_0 \to 0$, while $t_{1/2} \to \ln 2/k_e$.

The other proof of the existence of the induction step in peptide binding to MHC proteins could be an experimental observation of a zero slope $d[C(t)]/dt = 0$ at initial instant of time $t = 0$. It follows directly from Eqs. (1) and (2) and the initial conditions for this system. An experimental determination of a zero derivative at initial instant of time may be rather difficult because of a weak value of the detection signal which is proportional to the concentration of peptide–MHC complexes and starts from zero. Nevertheless, this phenomenon was observed by Sadegh-Naseri and McConnell [2] (Fig. 1a,c of this article), though the authors did not give any interpretation of the fact.

There is one more possible indication that peptide–protein association is not a simple binding reaction which is based on the experimental data of Witt and McConnell [1]. Really, based on Fig. 2, $t_{1/2}$ increase for a simple binding reaction in Eq. (3) $t_{1/2,max}/t_{1/2}$ $(P_0 \to 0)$ does not exceed 1.44, while the experimental observation gives about three-fold increase in half time of complex formation. But the significance of this argument is diminished by the controversy between the experimental data and the analytical prediction on the location of the maximum on $t_{1/2}$ curve.

3. Conclusions

The assumed kinetic model of peptide binding to MHC proteins gives us qualitative understanding of what experimental data should be expected according to this model and enables us to explain consistently the 'negative' $t_{1/2}$ plot phenomena. An analysis of the two-step mechanism of peptide–MHC binding indicates the following features of this system.

(1) The bell-shaped half-time curves as a function of peptide concentration is the feature of this kinetic model. Both a bell-shaped half-time curve or a 'regular' curve with $t_{1/2}$ decreasing as a function of the initial peptide concentration can be observed in the binding experiment. The result depends on the ratio of the equilibrium dissociation constant of the peptide–protein complex to the initial concentration of protein K_d/M_0. When K_d/M_0 is less than a certain value, the shorter $t_{1/2}$ at lower added peptide concentrations should be observed in the experiment.

(2) A simple one-step binding mechanism M + P = C also yields bell-shaped half-time plots for peptides with relatively high affinity to protein ($K_d/M_0 < 1 - 1/(2 \ln 2)$. Thus, an experimental observation of a bell-shaped half-time curve is not a proof of the existence of an induction step in peptide–MHC binding.

(3) An experimental study of peptide reaction with MHC protein at high peptide concentrations shows that $t_{1/2}$ stops decreasing (reaches nonzero asymptotic value) with the increase of peptide concentration. This result definitely confirms the existence of the induction step in peptide binding to MHC protein, which becomes rate limiting for high peptide concentrations.

(4) The location of the maximum on half-time graphs for the assumed two-step binding mechanism (Eqs. (1) and (2)) should occur at peptide concentration less or of the same order of magnitude as protein concentration. According to the experimental data of Witt and McConnell [1], the location of the maximum on the half-time curve occurs at peptide concentration much greater (about 400 times) than that of protein. This indicates that either more complicated mechanism than two-step binding is involved in peptide–MHC reaction, or that these experimental data are not quite accurate.

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Appendix A. On the numerical simulation of the kinetics of peptide–protein binding

Witt and McConnell [1] tried to obtain 'negative' $t_{1/2}$ plot from a numerical solution of the system of kinetic equations for the reactions in Eqs. (1) and (2). The following values of the parameters were used in the simulations: $M_0 = 1\ \mu M$, $k_1 = 1\ M^{-1}\ s^{-1}$, $k_2 = k_e = 3 \cdot 10^{-5}\ s^{-1}$ (which corresponds to the half time of the release of an endogenous peptide $\ln 2/k_e = 6.38$ h), and $P_0 = 5$, 25, 50 and 200 μM. With these values of the parameters, the curves for $[C(t)]$ were obtained from Figure 5 of Ref. [1]) which resembled the experimental data but did not yield 'negative' half-time plot. 'Negative' $t_{1/2}$ curve for a simple binding reaction in Eq. (3) was not observed; also in the numerical simulation with the same values of k_1 and k_2. In this case, $K_d/M_0 = 30$ and 'regular' decrease of $t_{1/2}$ with the increase of peptide concentration is consistent with Eq. (7).

Nevertheless, in order to obtain 'negative' half-time plots, Witt and McConnell [1] increased the value of the on-rate constant k_1 1000 times, which is very unrealistic value. At that point, their simulations became sensitive enough to yield 'negative' $t_{1/2}$ slope, which, as they affirm, is consistent with the observed experimental three-fold increase in $t_{1/2}$, though they still did not get 'negative' $t_{1/2}$ curve for a simple binding reaction in Eq. (3). Based on Eq. (7), the bell-shaped $t_{1/2}$ curve appears if K_d/M_0 is less than $r_c = 1 - 1/(2 \ln 2) = 0.2787$. For the values of the parameters used in this case $K_d/M_0 = 0.03$ and thus, 'negative ' half-time curve definitely exists in this case. To plot Fig. 1 for a simple binding reaction (which demonstrates the bell-shaped curve) we used exactly the same values of M_0, k_1 and k_2 for the curve A as in the numerical simulation mentioned above. It is very useful to have any analytical result to verify the correctness of a numerical simulation. If the authors of Ref. [1] obtained analytical equations for the kinetics of a simple

binding reaction in Eq. (3), they would see the discrepancy between the numerical simulation and the analytical calculation, and thus, would be aware that the computer program they used was not precise enough.

For that high value of k_1 (1000 M^{-1} s^{-1}) the time scale $(k_1P_0 + k_2)^{-1}$ of the Eq. (2) (100 s for $P_0 = 10$ μM) is much shorter than the time scale k_e^{-1} (9.3 h) of the reaction in Eq. (1). In this case, Eq. (8) which describes the kinetics of the reactions in Eqs. (1) and (2) at high peptide concentrations becomes the function of only the rate constant k_e, $[C(t)] = M_0[1 - \exp(-k_e t)]$, so that the release of an endogenous peptide is the limiting stage of binding. The numerical simulations in this case should not give any distinction for $P_0 \gg M_0$, which does not correspond to the experimental observations of Witt and McConnell [1] for $P_0 = 10$, 25, 100, and 200 μM and $M_0 = 0.25$ μM. It is also difficult to make a reasonable determination of that high value of the on-rate constant k_1 by comparison the numerical simulation results and the experimental data. The results of the numerical simulations in this case, will be very insensitive to the changes of k_1. This is especially obvious for the region of high peptide concentrations, where $[C(t)]$ practically does not depend on k_1 at all. Therefore, there is no definite proof that the value of k_1 which leads to the 'negative' $t_{1/2}$ in the numerical simulations, corresponds to the real value of the rate constant. But the most important is that 'negative' $t_{1/2}$ slope obtained through the numerical simulations occurred in the region of peptide concentrations $P_0 \leq M_0$, while in the experiment 'negative' half-time curve was observed at $P_0 \leq M_0$. This discrepancy between the results of the numerical simulations which yield 'negative' $t_{1/2}$ plot and the experimental data is not discussed in Ref. [1].

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