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On the requirement of oxidizing reagent for the formation of a disulfide bond

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Abstract

Cyclization of a peptide through the formation of a disulfide bond between the SH groups of cysteines on the *N*- and *C*-terminals of peptide was studied in degassed water solution under vacuum. Cyclization went to completion although the solution was oxygen deficient (the number of oxygen molecules available for the reaction was at least 16 times less than the number of peptide molecules). This result indicates that, contrary to the common assumption, disulfide bond formation does not necessarily require an oxidant (O_2, I_2, etc.) to occur. © 2001 Published by Elsevier Science B.V.

Keywords: Disulfide bond formation; Air oxidation; Cyclic peptide

1. Introduction

The reaction of disulfide bond formation in liquid solutions has been studied intensively for various conditions [1,2]. It was concluded that this reaction requires some oxidizing agent such as O_2 or I_2, and is very much promoted under basic conditions and also in the presence of a catalyst, such as ions of heavy metals. Disulfide bond formation occurs as:

$$2RSH + 1/2O_2 = RSSR + H_2O$$

$$2RSH + I_2 = RSSR + 2HI.$$

It seems that this process needs an oxidant to take the electron from the electronegative sulfur. If the two SH groups are in close proximity and the protons dissociate from both SH groups, the two negatively charged sulfurs will be repulsed and disulfide bond formation will not take place. Oxygen (or I_2) may attract the electrons from

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sulfurs to form H_2O (or HI), thus promoting formation of the disulfide bridge.

In our recent article [3], we studied the kinetics of peptide cyclization in water through disulfide bond formation between the SH groups of cysteines on the C- and N-termini of a peptide. It was observed that the rate of cyclization was strongly dependent on the temperature of the solution. For example, at pH 7, it takes 43.9 h to cyclize half of the peptide at 23°C. The same yield of cyclic peptide is obtained in 28.6 h at 37°C, while at 60°C, only 1.7 h is required. Kinetic data indicated that the formation of cyclic peptide most likely occurs through an intermediate step, at which the peptide forms a ring, so that the SH groups of the cysteines come into close proximity. A disulfide bond then forms, or the ring opens and the peptide retains its linear configuration. It was found that in basic media (pH 9–10) at 37°C, cyclization goes virtually to completion in less than 1 h.

The goal of this work was to determine if peptide cyclization in water solution really requires some oxidizing reagent to promote disulfide bond formation, or if this reaction may occur without an oxidizing reagent. We, therefore, tried to find out what kind of reaction is an adequate description of disulfide bond formation in the cyclization process — is it an oxidation or a release of two hydrogens as an overall result of some complicated reaction mechanism. In other words, we attempted to figure out which reaction, (1) or (2) below, corresponds to the formation of a disulfide bond

$$SH + SH = S{-}S + H_2 \quad (1)$$

$$SH + SH + 1/2O_2 = S{-}S + H_2O. \quad (2)$$

2. Description of the experiment

If air oxidation [Eq. (2)] is required for this reaction, then removing oxygen from the water solution should slow or even terminate the reaction. To study the cyclization we used the peptide (CSAEYYNKQYLERTRAELDTAC–$CONH_2$, m.w. = 2627) at a concentration p_o of 1 mg/ml, which corresponds to $p_o = 3.8 \times 10^{-4}$ mole/l. To check which reaction mechanism is correct, the following experiment was performed. The reaction vial was attached to a vacuum line and the liquid degassed. The equilibrium concentration of O_2, which was contained in the air exposed water solution at room temperature and 1 atm ambient pressure, was $c_o = 3.2 \times 10^{-4}$ mol/l [4], the volume, V, of the reaction solution in the experiment was 0.5 cm^3, and the surface, S, of the liquid was approximately 0.3 cm^2. The vacuum level in the experiment was approximately 25 torr (0.033 atm). That gives the equilibrium concentration of O_2 in the solution kept under vacuum $u_o = c_o \times 0.033 = 0.11 \times 10^{-4}$ mol/l [the solubility of gas in liquid is proportional to its partial pressure (Henry's law)]. Thus, the concentration of O_2 in the solution kept under vacuum is 36 times less than peptide concentration, so that if mechanism (2) took place, we would not have enough O_2 to cyclize all peptide in the degassed solution.

The peptide was added and cyclization was monitored (by chromotographical analysis of the aliquots) while the solution was kept under vacuum. We conducted the cyclization for 1 h at pH 10 (adjusted by NH_4OH) and 40°C using a heating block, which brings the reaction vessel to 40°C in a few minutes. As studied recently [3], the reaction under these conditions in the air exposed solution was fully complete in less than 1 h.

When peptide was added to the reaction vial, it was exposed to an ambient pressure for approximately 10 s. At that point, we needed to estimate how much oxygen was dissolved in the solution while it was exposed to the air. We also needed to estimate how much oxygen would go into solution during the reaction, because the vacuum was not absolute, so that there might still be a diffusion flow of ambient oxygen into the solution if oxygen was used for cyclization. If these two factors did not lead to a significant increase in oxygen concentration in the reacting system, then our experiment would be consistent. The calculations considered in Appendix A showed that the maximum quantity of oxygen available during the reaction could cyclize no more than 13% of the peptide, if

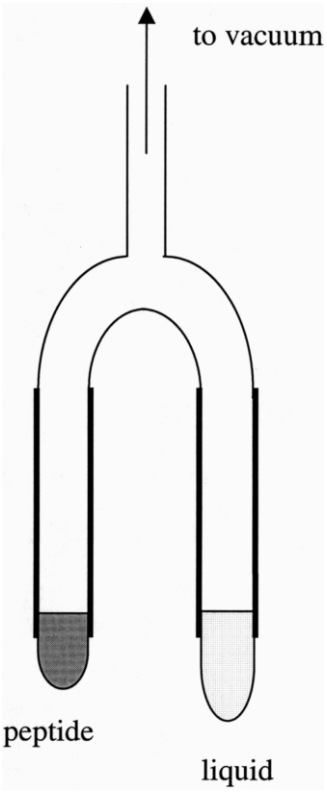

Fig. 1. Diagram of the experimental device used for the studies of peptide cyclization under vacuum.

only an oxygen mediated oxidation mechanism [Eq. (2)] takes place. In order to obtain more convincing results, we also performed cyclization studies using the system where the peptide was added to a degassed solution under vacuum (in this case, we eliminate the oxygen intake into the solution at the beginning of the experiment and do not need to estimate how much oxygen would be dissolved when the reaction vessel was opened to add a peptide). The experimental device is shown schematically in Fig. 1. After keeping the system under vacuum, it was tilted to transfer the degassed liquid to the peptide. There was no significant difference in the experimental results, whether a peptide was added to the liquid under vacuum or by quick opening of the reaction vessel. This confirmed the validity of the calculations in Appendix A which predicted a low intake of the oxygen into the solution.

Peptides were analyzed by reverse phase HPLC on a C18 analytical column (5μ, 300 A, 150 × 2.1 mm, Vydac) using a water–acetonitrile gradient with 0.01% v/v TFA in each solvent. A linear gradient from 10% to 38% of acetonitrile in 22 min was applied. Under these conditions, the difference in retention times for the cyclic and linear peptides was quite substantial: 15.4 min for the cyclic peptide and 17.9 min for the linear one; a typical chromatogram is shown in Fig. 2. Cyclization was also confirmed by mass spectra analysis of the peptides.

3. Results and discussion

One hour after the linear peptide was added to the degassed solution and then kept under vacuum, an aliquot was taken and immediately analyzed by HPLC. There was only one peak on the chromatogram, which corresponded to the cyclic peptide, indicating complete cyclization. Thus, we come to an important conclusion, that in a strongly alkaline solution (pH 10), the formation of a disulfide bond, which leads to peptide cyclization, does not require oxygen (or other oxidizing reagent) and it definitely follows mostly mechanism (1), but not (2). We cannot completely eliminate the oxidation reaction [Eq. (2)] as a possible mechanism of cyclization based on the experiment performed, but it cannot provide more than 13% of the cyclic peptide yield, while the remaining 87% cyclizes via Eq. (1), which represents some alternative mechanism of disulfide bond formation.

To obtain more details about the mechanism of disulfide bond formation, we studied the kinetics of cyclization at room temperature in the neutral (pH 7) and basic (pH 10.6) solutions. The experiment was conducted in the following way. One reaction tube was attached to the vacuum line (0.033 atm) and cyclization kinetics was monitored while the solution was kept under vacuum. Nearby, cyclization of the peptide at the same concentration, but in the tube exposed to the ambient pressure, was performed. We used the same concentration of peptide as before, i.e. 1 mg/ml. Both vials were always at the same room

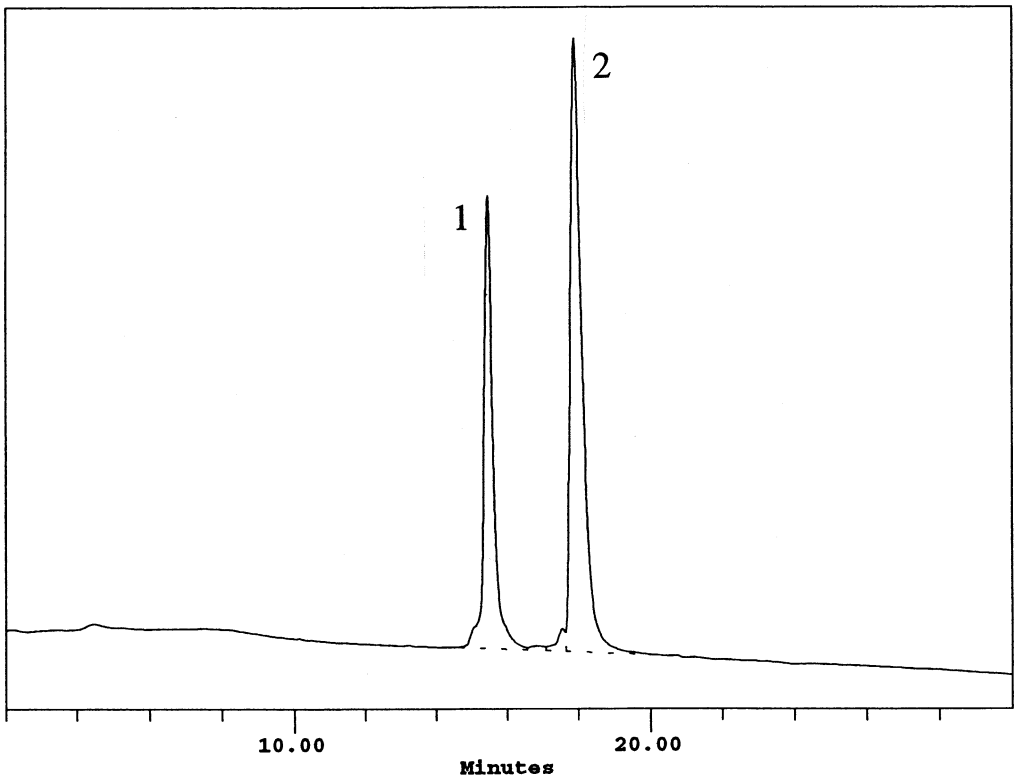

Fig. 2. Chromatogram of disulfide bridged (cyclic) and linear peptides: (1) cyclic peptide; (2) linear peptide.

temperature and, thus, we need not keep the temperature strictly constant to compare the reaction rates. The kinetic data for cyclization are shown in Figs. 3 and 4.

These experiments provide very useful information: there is virtually no change in the rate of disulfide bond formation in the basic solution (Fig. 3), and there is a substantial rate decrease of the process in the neutral one (Fig. 4).

Let us discuss the kinetics of peptide cyclization in the basic solution (Fig. 3). For the system kept under vacuum, when there is not enough oxygen to complete the reaction via oxidation mechanism (2), cyclization proceeds virtually at the same rate as in the solution which is saturated with oxygen at ambient pressure. It means that disulfide bond formation under these conditions does not follow a traditional oxidation mechanism, but rather occurs through an alternative path as $SH + SH = S{-}S + H_2$, which is strongly promoted by basic media. As we studied recently [3], the kinetics of peptide cyclization is well described by a two-step mechanism

$$P_L \underset{k_2}{\overset{k_1}{\rightleftarrows}} P^* \tag{3}$$

$$P^* \overset{k_3}{\rightarrow} P_C \tag{4}$$

where P_L and P_C are the notations for the linear and cyclic peptides, respectively, and P^* indicates an intermediate conformation, which either unfolds or reacts irreversibly to form cyclic peptide. Reaction (3), which occurs due to Brownian motion, is essentially reversible, it shows the formation of an intermediate state product P^*, k_1 and k_2 are the on- and off-rate constants of this reaction. Reaction (4) proceeds in only one direction according to experimental data, which con-

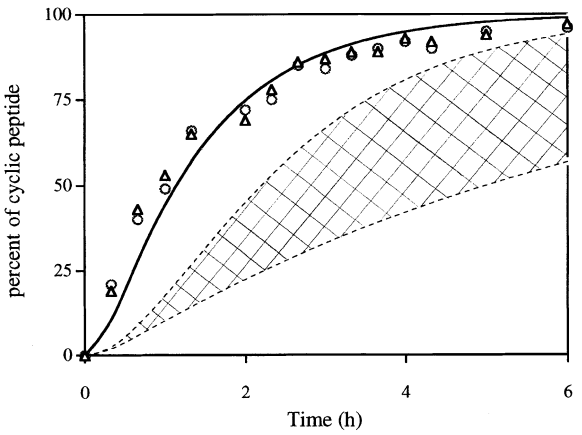

Fig. 3. Kinetics of peptide cyclization through the formation of a disulfide bond at room temperature in basic solution (pH 10.6): △ — time points for the air exposed solution, solid line is the experimental data fitted by Eq. (5) with $b_1 = 4\ h^{-1}$, $b_2 = 0.8\ h^{-1}$, ○ — time points for the solution kept under vacuum. Shaded area is the anticipated rate of cyclization under vacuum, if it would only follow air oxidation mechanism.

firm that cyclization goes to completion, k_3 is the rate constant of this reaction. The initial conditions for this system are $[P_L] = p_o$ and $[P^*] = [P_C] = 0$ at $t = 0$. The solution of formal chemical kinetics for Eqs. (3) and (4) (details are given in Appendix B) is:

$$[P_C](t)/p_o = 1 - [b_1\exp(-b_2 t) - b_2\exp(-b_1 t)]/(b_1 - b_2) \quad (5)$$

where

$$b_{1.2} = \left\{k_1 + k_2 + k_3 \pm \left[(k_1 + k_2 + k_3)^2 - 4k_1k_3\right]^{1/2}\right\}/2, \quad (6)$$

where b_1 and b_2 correspond to the positive and negative signs, respectively. According to Eq. (6), $b_1 + b_2 = k_1 + k_2 + k_3$ and $b_1 b_2 = k_1 k_3$. It is interesting to note that Eqs. (3) and (4) are mathematically identical to the equations of the Michaelis–Menten model for enzymatic catalysis. However, we did not use a steady state condition for an intermediate P^*, which leads to a more complicated biexponential solution [Eq. (5)]. From Eq. (6), it also follows that $b_2 \le k_3 \le b_1$. The experimental data for peptide cyclization in the solution exposed to the ambient pressure in Fig. 3 are fitted by Eq. (5) with $b_1 = 4\ h^{-1}$ and $b_2 = 0.8\ h^{-1}$.

Let us assume that disulfide bond formation in the solution either exposed to the ambient pressure or kept under vacuum occurs only as air oxidation reaction, so that Eq. (4) is $P^* + 1/2O_2 \rightarrow P_C + H_2O$. The rate constant k_3 of this reaction (defined as $dP^*/dt = -k_3 P^*$) then depends on the oxygen concentration $k_3 \sim [O_2]^{1/2}$. When cyclization is conducted under vacuum (30 times decrease of the ambient pressure), k_3 should decrease compared to its value for the air exposed system, $k_3 \rightarrow k_3/(30)^{1/2}$ (we assume here that there is oxygen saturation of the liquid under vacuum, which is the most favorable condition for the oxygen supply, otherwise oxygen diffusion into the solution would effectively lead even to greater decrease of k_3 value). With this change of the rate constant k_3, the rate of cyclization [Eq. (5)] should drop substantially and would be in the shaded area in Fig. 3 (similar results for a strong decrease in cyclization rate also follow from the simplest one-step cyclization model: $P_L + 1/2O_2 \rightarrow P_C + H_2O$, which is, however, not as accurate in reproducing experimental data as the two step

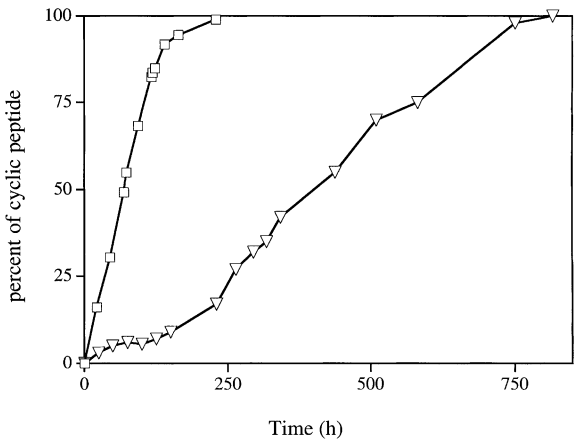

Fig. 4. Kinetics of peptide cyclization through the formation of a disulfide bond at room temperature in neutral solution (pH 7): □ — time points for the air exposed solution, ▽ — time points for the solution kept under vacuum.

model). However, this prediction for a slow down of cyclization rate (in the case of oxidation mechanism) was not observed. The rate of the process practically did not change, which is an indication that air oxidation is negligible in this experiment and non-oxidation disulfide bond formation [Eq. (1)] dominates.

For the neutral solution, an assumption that cyclization reaction occurs with the decrease of volume cannot explain a strong decrease in the rate of the process. Really, the reaction rate constant k is proportional to $\exp(-\Delta E/RT)$, where the activation energy of the reaction ΔE includes the work $P\Delta v$ against the ambient pressure P, and Δv here is the volume change of the reaction. Then $\partial k/\partial P = -k\Delta v/(RT)$. According to the experimental observation, the rate of cyclization increases with the increase of ambient pressure, i.e. $\partial k/\partial P > 0$ and, therefore, $\Delta v < 0$. Taking into account that Δv does not exceed 18 ml/mole (this is just the volume of 1 mole of water), we can easily obtain that the change in the rate constant k is less than 0.1% when the ambient pressure changes from 0 to 1 atm. Therefore, the change of the reaction rate due to possible volume decrease is negligible. The observed 5–6 times decrease of the cyclization rate (Fig. 4) is approximately the same as a $(30)^{1/2}$ times decrease of the rate constant. The diffusion of oxygen into the solution may also slow down cyclization process (time required to saturate the solution with oxygen is estimated as $L^2/(2D) \approx 20$ h, see Appendix A for details).

Thus, we conclude that cyclization under vacuum in the neutral solution goes more slowly, because by removing oxygen from the solution, we reduce the contribution of the reaction of air oxidation [Eq. (2)] to the total rate of the process. In principle, both mechanisms of disulfide bond formation (1) and (2) may take place in the neutral solution, but traditional air oxidation [Eq. (2)] is definitely a preferable way, while a non-oxidation mechanism is negligible. On the contrary, under basic conditions, non-oxidation kinetics of disulfide bond formation take place and dominates over air oxidation. The rate of the process is much faster (compare Figs. 3 and 4) in this case.

Kinetic study does not give a direct clue to the mechanism of non-oxidation formation of a disulfide bond. The transition from reagents to products (especially for reactions with electron transfer in liquid solution) is a very complicated process. Possibly NH_4^+ ions in our experiment act as a catalyst in the same way as ions of heavy metals [1,2] as studied earlier, though cyclization through the formation of a disulfide bond does not require oxygen. It seems likely, that in aqueous solution, solvation of the NH_4^+ ions around negatively charged sulfurs leads to the change of the electron density and effectively decreases the bonding of the electrons to it. Thus, the energy barrier of disulfide bond formation becomes lower, which enables a transition of the linear peptide to the thermodynamically more preferable cyclic form with the release of H_2 molecule. It could be a subject of a separate study on how different ions influence the cyclization process in an oxygen deficient solution. The cyclization rate and yield of cyclic peptides were observed to be dependent on the oxidizing reagents [O_2, H_2O_2, I_2, $K_3Fe(CN)_6$] used for cyclization [5]. It is possible that oxidizers may also influence the cyclization process indirectly by changing the energy barrier of Reaction (2).

Appendix A

Let us evaluate how much oxygen will go into the solution while the reaction vessel is opened to add peptide. At 22°C, the equilibrium concentration of oxygen O_2 in water is $c_o = 3.2 \times 10^{-4}$ M [4]. We used $V = 0.5$ ml of solution for kinetics studies, which thus contains 9.6×10^{16} molecules of oxygen. For the vapor, the impinging rate β, which is defined as the number of molecules which hit the unit surface in a unit time, is given by the equation [6]:

$$\beta = P/(2\pi mkT)^{1/2},$$

where P is the gas pressure, m is the mass of the molecule, k is Boltzmann's constant, and T is absolute temperature. The number of molecules that hit the surface, S, in a unit time is βS. In our

experiment, the surface of the liquid in the vial was approximately $S = 0.3$ cm^2. The oxygen pressure is 0.23 atm (ambient air contains approx. 23% of oxygen). Then we get $\beta S = 1.9 \times 10^{22}$ molecule/s, so that the number of molecules which hit the surface of the solution in 1 s is much greater than the number of oxygen molecules in the solution at saturation. It means that the delivery of oxygen is very fast, thus the surface of the degassed solution will be saturated with oxygen virtually immediately after the reaction vessel is opened. It also means that diffusion of oxygen into the solution will be the limiting step in saturating the liquid with oxygen.

To estimate how much oxygen will go into solution, we need to solve the one-dimensional diffusion equation, which corresponds to the conditions of the experiment and then calculate the flow of oxygen into the solution as a function of time. We consider an opened reaction vessel as a cylinder with the surface at coordinate $x = 0$ and the bottom at $x = L$. The diffusion flow of gas inside the liquid is given by the equation:

$$\mathbf{j} = -D\partial c/\partial x, \tag{7}$$

where D is the diffusion coefficient of oxygen molecules in the liquid and c is their concentration as a function of coordinate x and time t, $c = c(x,t)$. According to the law of conservation of mass $\mathrm{div}\mathbf{j} + \partial c/\partial t = 0$ and, thus, we have a well-known diffusion equation:

$$\partial c/\partial t = D\partial^2 c/\partial x^2. \tag{8}$$

The initial condition for Eq. (8), which corresponds to the experiment is:

$$c(x,0) = u_o \; 0 < x < L \tag{9}$$

where u_o is the initial concentration of oxygen in the degassed solution (supposedly considerably less than that for the air exposed liquid).

The boundary conditions for this system are:

$$c(0,t) = c_o \tag{10}$$

$$c(x,\infty) = c_o \tag{11}$$

where c_o is the equilibrium concentration of oxygen in the solution exposed to the air (in the experiments performed $u_o = c_o \times 0.033 = 0.11 \times 10^{-4}$ M). Eq. (10) means that we assume the surface of the liquid to be saturated at any instant of time and Eq. (11) means that the solution becomes uniformly saturated at infinitely long time. The diffusion equation [Eq. (8)] has a general solution [7]:

$$\begin{aligned} c(x,t) = &\sum_{n=1}^{\infty} (A_n \sin\lambda_n x + B_n \cos\lambda_n x) \\ &\times \exp(-\lambda_n^2 Dt) + Hx + G \\ &+ Ft^{-1/2}\exp(-x^2/(4Dt)) \end{aligned} \tag{12}$$

The coefficients A_n, B_n, λ_n, H, G and F in Eq. (12) can be found [8] using initial and boundary conditions [Eqs. (9)–(11)]. The final result in our case is:

$$\begin{aligned} c(x,t) = &4\pi^{-1}(u_o - c_o) \sum_{k=0}^{\infty} \sin(\pi(2k+1)x/L) \\ &\times \exp\left(-\pi^2(2k+1)^2 Dt/L^2\right) + c_o \end{aligned} \tag{13}$$

To find the quantity of oxygen $J(t)$ which gets into the liquid when the reaction tube is opened during the time interval t, we need to calculate:

$$\begin{aligned} J(t) &= \int_0^t j(t')S\mathrm{d}t', \\ j(t') &= -D\partial c(x,t')/\partial x|_{x=0} \end{aligned} \tag{14}$$

Using Eq. (13) and substituting the summation over k by integration we obtain the following equation for the flow $j(t')$:

$$\begin{aligned} j(t') = &2(c_o - u_o)(D/\pi t')^{1/2} \\ &\times \left[1 - \mathrm{erf}\left(\pi(Dt')^{1/2}/L\right)\right], \end{aligned} \tag{15}$$

where $\mathrm{erf}(x) = 2/\pi^{-1/2}\int_0^x \exp(-z^2)\mathrm{d}z$ is the error function.

In our case, the diffusion coefficient of oxygen O_2 in water at 40°C is $D = 3.24 \times 10^{-5}$ cm^2/s [9],

$L = 1.7$ cm and t is approximately 10 s, which is the time needed to add the peptide to the solution (or take an aliquot) and reattach the reaction vial to the vacuum line. With these values, the error function in Eq. (15) is much less than 1, and eventually we obtain from Eq. (14) the quantity of oxygen which will get into solution while peptide is added or an aliquot is taken:

$$J(t) = 4(c_o - u_o)S(Dt/\pi)^{1/2} \tag{16}$$

The quantity of oxygen contained in the saturated air exposed solution equals to c_oV. Using the given values of all parameters, we finally have:

$$J(t = 10\text{ s})/c_oV = 0.024. \tag{17}$$

To estimate how much oxygen J_1 will get into the system during the reaction, we need to find the oxygen flow from the saturated surface with concentration u_o into the solution. The surface will still be kept saturated with oxygen at equilibrium concentration u_o, even at low pressure $P = 25$ torr, because of a high impinging rate β ($\beta S \gg u_oV$). We will overestimate the intake of the oxygen into the reaction system if we assume that the diffusion goes into the solution, which initially does not contain oxygen at all. Then the diffusion flow will be given by Eq. (15) with u_o instead of $(c_o - u_o)$:

$$j(t') = 2u_o(D/\pi t')^{1/2}\left[1 - \mathrm{erf}\left(\pi(Dt')^{1/2}/L\right)\right]. \tag{18}$$

We again overestimate the flow of oxygen into the solution if we neglect the error function in Eq. (18) (take it as 0), and then according to Eq. (14) we get:

$$J_1(t) = 4u_oS(Dt/\pi)^{1/2}.$$

For $u_o = 0.033c_o$ at room temperature (this is also an overestimation because at 40°C, the saturated oxygen concentration is lower than at 23°C), we get:

$$J_1(1\text{ h}) = 0.015c_oV. \tag{19}$$

Thus, the overall quantity of oxygen that can get into the solution when peptide was added [Eq. (17)], and during the reaction [Eq. (19)], will be:

$$J(t = 10\text{ s}) + J_1(1\text{ h}) = 0.039c_oV$$

and the total quantity of oxygen that was available during the reaction is:

$$J(t = 10\text{ s}) + J_1(1\text{ h}) + u_oV = 0.072c_oV.$$

According to the stoichiometry of peptide oxidation by traditional mechanism [Eq. (2)], we need 0.5 mole of oxygen O_2 to convert 1 mole of linear peptide into the cyclic one by forming a disulfide bridge between two cysteines. Therefore, the quantity of oxygen available during the reaction would not be able to yield more than $0.144c_oV$ moles of cyclic peptide, which is at least eight times less than the number of moles p_oV of initially dissolved linear peptide ($p_oV/0.144c_oV = 8.25$). The above calculation brings us to an important conclusion: we do not have enough oxygen in the solution during the experiment to take the reaction to completion via mechanism (2). No more than 13% of peptide will cyclize if only this reaction mechanism is involved.

Appendix B

Let us obtain the solution of the following kinetic model for peptide cyclization:

$$P_L \underset{k_2}{\overset{k_1}{\rightleftarrows}} P^* \tag{20}$$

$$P^* \xrightarrow{k_3} P_C \tag{21}$$

where P_L and P_C are the notations for the linear and cyclic peptides and P^* indicates an intermediate conformation, which either unfolds or reacts irreversibly to form cyclic peptides. The initial conditions for this system are:

$$[P_L] = p_o,\ [P^*] = [P_C] = 0 \text{ at } t = 0 \tag{22}$$

where t is time.

The formal chemical kinetics equations for this system, according to Eqs. (20) and (21), are:

$$d[P^*]/dt = k_1[P_L] - (k_2 + k_3)[P^*] \quad (23)$$

$$d[P_C]/dt = k_3[P^*]. \quad (24)$$

According to the law of conservation of mass $[P_L] = p_o - [P_C] - [P^*]$. Inserting this equation for $[P_L]$ into Eq. (23), we obtain the system of two differential equations for the variables $[P^*]$ and $[P_C]$:

$$d[P^*]/dt = -k_1[P_C] - (k_1 + k_2 + k_3)[P^*] + k_1 p_o \quad (25)$$

$$d[P_C]/dt = k_3[P^*]. \quad (26)$$

It is convenient to solve this system applying the Laplas transform method [7] for the variables:

$$F(s) = L[f(s)] = \int_0^\infty f(t)e^{-st}dt,$$

then Eqs. (25) and (26) take the form:

$$s[P^*](s) = -k_1[P_C](s) - (k_1 + k_2 + k_3)[P^*](s) + k_1 p_o/s \quad (27)$$

$$s[P_C](s) = k_3[P^*](s). \quad (28)$$

Solving Eqs. (27) and (28) to find $[P_C](s)$, we get:

$$[P](s) = k_1 k_3 p_o/[s(s + b_1)(s + b_2)], \quad (29)$$

where $b_{1.2} = \{k_1 + k_2 + k_3 \pm [(k_1 + k_2 + k_3)^2 - 4k_1k_3]^{1/2}\}/2$, b_1 and b_2 corresponds to the positive and negative signs, respectively.

Using the inverse Laplas transform for Eq. (29) we finally obtain:

$$[P_C](t)/p_o = 1 - [b_1 \exp(-b_2 t) - b_2 \exp(-b_1 t)]/(b_1 - b_2).$$

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