Antagonistic Enzymes May Generate Alternate Phase Transitions Leading to Ephemeral Gels

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ABSTRACT In some biological processes, two enzymes with antagonistic activities—the one creating a bond, the other destroying it—are involved in a reaction cycle. Several catalysts have the ability to modify the rheological properties of biological media participating in the production of a solid gel phase which later dissolves. Transglutaminase, catalyzing intermolecular protein cross-linking, is considered here as a reverse protease as far as the physical state of a proteic gel is concerned. A kinetic model including diffusion constraints and based on a protease/transglutaminase cycle interconverting insoluble gel and soluble proteolysis fragments showed that alternate sol/gel and gel/sol transitions could occur within such a system, generating transient gel phases. Then, ephemeral gels were obtained in vitro using an experimental system consisting of gelatin, transglutaminase, and thermolysin. Modulating the enzyme activity ratio allows us to "program" the global behavior: polymerization/solubilization cycle of a mixture containing at least one protein and two enzymes without any change in temperature or medium composition.

INTRODUCTION

Enzymes usually catalyze very specific reactions, often implying one single chemical group (1). However, in some cases, these subtle modifications at the molecular scale cause important variations at the supramolecular level. One example is milk coagulation induced by the hydrolysis of the single Phe^{105}-Met^{106} peptidic bond of κ-casein (2). Among the 3500 characterized enzymes, some are particularly efficient at catalyzing critical phenomena leading to phase transitions. These include proteases which may dissolve insoluble protein phases (3). In various physiological and pathological events, proteases modify the macroscopic organization of cell environment. The implication of proteases acting as destructive catalysts is of crucial importance in embryogenesis (4) as well as in neoangiogenesis and tumor dissemination (5,6). In contrast, proteases contribute to the generation of a new solid phase in wound healing or blood coagulation. In this case, they cleave only a very small number of peptide bonds to activate zymogens. This will generate a viscous solution or a soft gel phase which is later stabilized by transglutaminases which create covalent intermolecular protein cross-linking (7), giving rise to irreversible gel networks. In blood coagulation or wound healing, the two types of enzymatic reactions coexist and act synergistically (8,9); the proteolytic action of thrombin is followed by protein bonding due to Factor XIII, a transglutaminase. Both participate in the formation of a solid phase from macromolecules in solution.

However, in other biological events, these two enzymes can be considered as antagonistic ones as proteases cleave peptide bonds, whereas transglutaminase generate isopeptide bonds. In the extracellular matrix (ECM), these two types of enzymes act conversely. ECM properties are an essential regulator of cell physiology and behavior; and during normal maintenance or pathological modifications, cell-controlled composition changes result in macroscopic modifications defined as ECM remodeling (10,11); this complex process involves both proteases and transglutaminases. In the ECM, the major rationale is that proteolysis would disorganize ECM assembly in a way that makes it permeable to cells (12). In agreement with this hypothesis, numerous ECM-degrading proteases have been correlated with tumor invasiveness or metastatic potential (5,6). Contrarily, transglutaminase contributes to the insolubilization of the protein lattice and is accordingly implied in ECM remodeling (11). In other words, whereas protease disorganizes the ECM via partial solubilization (3), transglutaminase contributes to reorganizing it, creating insoluble matrix from soluble fragments. Enzyme-ECM interactions form a complex and nonlinear system that can manifest nontrivial or emergent properties. Oscillating concentrations for soluble proteins and insoluble lattices were calculated in a model considering proteases and transglutaminases as reverse catalysts of a futile cycle in which ECM proteins and their soluble proteolysis fragments are interconverted (13).

Until now, to our knowledge no attempt to experimentally generate such alternate phase transitions in vitro by enzymes has been reported. In vitro, phase transitions are generally due to a change in the medium composition (e.g., concentration, ions, and pH) or in the physicochemical parameters (e.g., temperature and pressure), rather than to enzymes (14).

Here we describe the use of enzymes to catalyze in vitro sol/gel followed by gel/sol transitions to obtain the successive polymerization and solubilization of proteins without further addition of reactants and without any change in temperature or pressure. This presupposes the capacity to

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''program'' the global behavior of a mixture containing at least one protein and two enzymes. We first focused on the kinetics of antagonistic enzymes, elaborating a mathematical model based on a transglutaminase/protease cycle interconverting soluble proteins and an insoluble network. This model allows the prediction of protein bonding/dissociation and of the resulting phase transitions.

Among the proteins able to give rise to a gel, gelatin was chosen as it has the ability to form thermally reversible networks (15). Below the sol/gel transition temperature, part of the protein coils gives rise to triple helices reminiscent of the native collagen (16) and protein solution turns into gel. Physical protein gels may be stabilized by the further addition of covalent bonds due to transglutaminase reaction, which also lead to chemical gels at high temperatures (17). Based on the model, we were able to obtain gelatin ephemeral gels experimentally, examples of which are described in this study.

MATERIALS AND METHODS

Materials

Gelatin used in this study is a kind gift from Rousselot; it was extracted from pig skin by an acidic process, has a pI of 8.74, a bloom of 292 g, an average molecular weight of 168,500, and a polydispersity of 1.91. Gelation properties are similar to those of Sigma (St. Louis, MO) Type A1 gelatin (G2500). Final concentration used was 5 g/100 mL^{-1} (5%, w/v) in 50 mM Tris HCl buffer, pH 7.4.

Ajinomoto Transglutaminase Activa WM from *Streptoverticillium sp.* was provided by Unipex (Paris La Défense, France). It was used without further purification, except for a filtration through a 0.2-μm membrane. The specific activity was determined according to de Macédo et al. (18). *Thermolysin* (protease type X from *Bacillus thermoproteolyticus rokko*, from Sigma P-1512) is a zinc metalloprotease. Enzyme activity was measured at 27°C and 40°C on a model substrate (*N*-(3-[2-furyl]acryloyl)-Gly-Leu-Amide, from Sigma f-7383). The calculated $Vi_{40°C}/Vi_{27°C}$ ratio is 1.66. All enzymes were stored at −20°C and solubilized extemporaneously to the desired concentration in 50 mM Tris-HCl buffer, pH 7.4.

Preparation of gels

Gelatin (10%, w/v) was swelled overnight at 4°C in buffer. Before use, this stock solution was incubated at 40°C for 30 min then diluted at 40°C to obtain a 5% solution. Various gels were prepared with different networks (19).

Physical gel

The 5% gelatin solution was incubated at 40°C during 155 min then cooled to 27°C with a temperature decrease of 0.5°C/min. To evaluate the gel time, time is measured from the beginning of the cooling ramp. No physical gel was obtained for temperatures above 34°C at concentrations up to 10%. This gel is not an equilibrium state and it continuously evolves with time. Raising back the temperature leads to a gel/sol transition (15,19,20).

Chemical gel

This gel is cross-linked by enzyme-catalyzed covalent bonds. One unit of transglutaminase was added to a 5% gelatin solution. The reaction was performed at 40°C. Under the same conditions, a 5% gelatin solution without enzyme shows no gelation. This gel is irreversible versus temperature.

Physicochemical gel

One unit of transglutaminase was added at 40°C to a 5% gelatin solution. That solution was directly cooled to 27°C where coils undergo conformational transition and form triple helices so that the ''physicochemical'' gel is due to both weak interactions and covalent bonds.

Rheology

Rheology measurements were performed with an AR 1000 from TA Instruments or on a RheoStress 150 from Thermo Electron (Thermo Scientific, Courtabeuf, France) operating in the oscillatory mode, with a strain of deformation of 1% and a frequency of 1 Hz. Deformation, storage modulus G', and loss modulus G'' were recorded as a function of time. Gel time was estimated when $G' = G''$. Temperature was controlled by a Peltier device. A cone/plate geometry with a cone of 6 cm/2° was used. Temperature ramps of 0.5°C/min^{-1} were applied.

Optical rotation

The amount of helices is derived from the specific optical rotation (21). Optical rotation was measured on a Perkin-Elmer 341 polarimeter (Perkin-Elmer, Foster City, CA). Temperature control was performed by a Julabo (Seelbach, Germany) FS18 bath. Cooling and heating ramps of 0/5°C/min^{-1} were applied.

The helix amount, χ, is derived from

$$\chi = \frac{[\alpha]_\lambda^{\text{helix}} - [\alpha]_\lambda^{\text{coil}}}{[\alpha]_\lambda^{\text{collagen}} - [\alpha]_\lambda^{\text{coil}}},$$

where λ is the wavelength ($\lambda = 436$ nm), $[\alpha]_\lambda = \alpha/lc$ is the specific optical rotation of the protein in solution, c is the concentration (grams per cubic centimeter), l is the optical path (0.1 decimeter), α is the optical rotation angle (degrees) measured experimentally, $[\alpha]_\lambda^{\text{collagen}}$ is the specific optical rotation of native soluble collagen ($\chi = 1$), which contains only triple helices, and $[\alpha]_\lambda^{\text{coil}}$ is the specific optical rotation of the coils ($\chi = 0$). ($[\alpha]_{436\text{nm}}^{\text{collagen}} = -800$ deg cm^3 g^{-1} dm^{-1} at 27°C, $[\alpha]_{436\text{nm}}^{\text{coil}} = -274$ deg cm^3 g^{-1} dm^{-1} at pH = 7.4 and 40°C (15)).

The additivity of the signals due to both transglutaminase and gelatin was checked. The signal from the enzyme, being constant, was subtracted from the apparent angle.

RESULTS

Elaboration of the kinetic model

We considered two enzyme reactions. The first is catalyzed by a transglutaminase (T) which binds soluble protein molecules (s) into a protein chain network (g). The second reaction is catalyzed by a protease (P) and solubilizes the bound proteins. In our model, protease and transglutaminase become the two antagonistic catalysts of a futile cycle between a protein network and its proteolysis fragments. On a larger scale, this futile cycle also represents a double phase transition: from sol to gel under the transglutaminase action and from gel to sol under the protease action. Moreover, the protease also hydrolyzes soluble proteins into fragments (f) which are too small to incorporate into the protein network, resulting in fragment efflux from the cycle. A schematic representation of our model is shown in Fig. 1 *A*. Below the sol/gel transition temperature, part of the protein coils gives rise to triple helices, reminiscent of how the native collagen and protein solution spontaneously turns into gel. When the

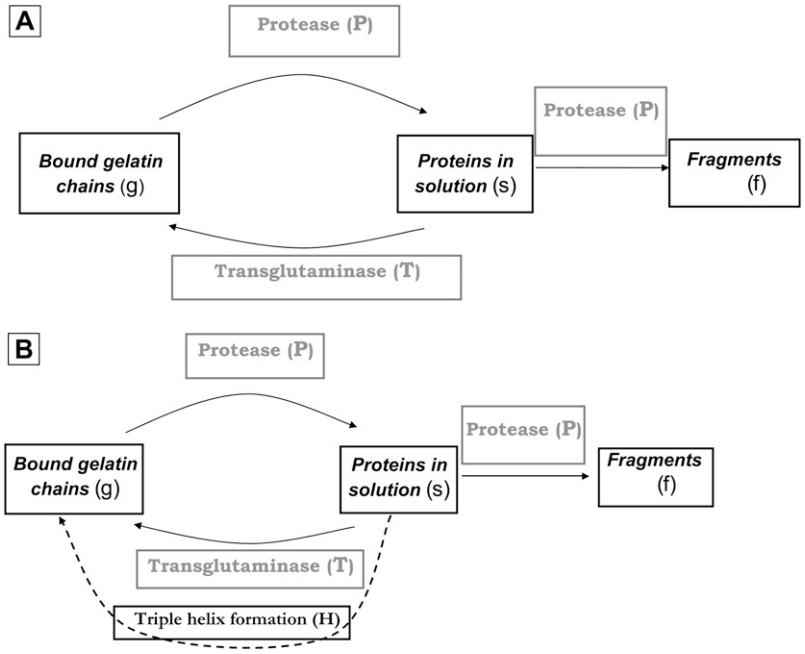

FIGURE 1 Futile cycle between a protein network and its proteolysis fragments using protease and transglutaminase as antagonistic catalysts. Reactions: *A* at 40°C and *B* at 27°C, including a nonenzymatic reaction.

enzymatic reaction was performed at 27°C—where coils undergo conformational transition and form triple helices so that a physicochemical gel due to both weak interactions and covalent bonds was obtained—the triple helix formation (nonenzymatic reaction) was added to the model (*dashed line* in Fig. 1 *B*).

Both enzymes are assumed to display Michaelis-Menten kinetics with k_p and k_T as the catalytic constants and K_P and K_T as the Michaelis constants for protease and transglutaminase, respectively. Protease-catalyzed degradation of the fragments is also taken into account. We assume that the kinetic constant for this reaction is lower than that for insoluble matrix protein proteolysis; this takes into account the multi-step process needed to obtain small fragments from a whole protein. The affinity of the protease is supposed to be identical for soluble and insoluble proteins (i.e., K'_p and K_P). P and T are the concentrations of protease and transglutaminase, respectively, which remain constant.

We have observed protein gelation under the action of transglutaminase through viscoelasticity measurements. When transglutaminase acts in a liquid phase, the inverse of the gel time (equivalent to gelation kinetics) is a direct function of enzyme concentration (Fig. 2). This first order kinetics upon enzyme concentration shows that despite the viscosity increase in the solution, the transglutaminase reaction is not diffusion controlled. However, our data show that the evolution of macroscopic properties is strongly reduced after the gel point (19). We estimated enzyme kinetics to be proportional to network formation (G' evolution) over 1 h after the gel point. At that stage, a pseudostationary state can be assumed, as only 18% of the possible substrate has been used (22) and the mechanism is cycling. However, a nonlinear relation is obtained in the gel phase between the enzymatic reaction and enzyme concentration (Fig. 3). This effect is characteristic of diffusional constraints. We consider here that as the covalent bonding increases inside the gel, the diffusion of the enzyme inside the protein network becomes increasingly limited. To take this diffusion reaction mechanism into account, the terms due to the transglutaminase reaction contain a progressive exponent (β) affecting transglutaminase concentration, with a value that varies with time from 1 to 1.85 as calculated from Fig. 4 and analogs with other transglutaminase concentrations. We consider having obtained a gel phase as soon as an 18% threshold of bound gelatin chains is reached as previously described (14).

V_H is estimated from polarimetry measurements (Fig. 5) and represents the evolution of triple helices with time, a nonenzymatic reaction. The apparition of helices was measured in a physical gel without enzyme at 27°C and in a gel

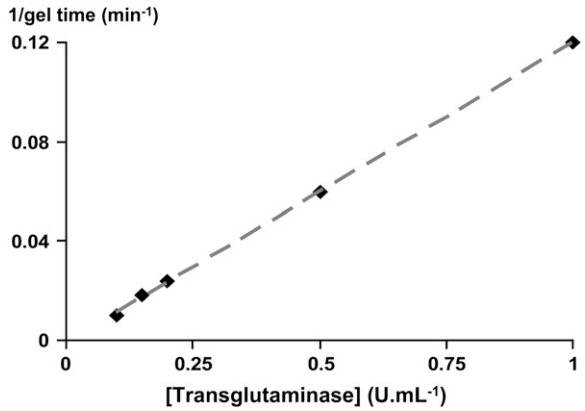

FIGURE 2 Gelation kinetics of 5% gelatin solutions at 40°C as a function of transglutaminase concentration $y = 0.12x - 0.0018$, $R^2 = 0.99$.

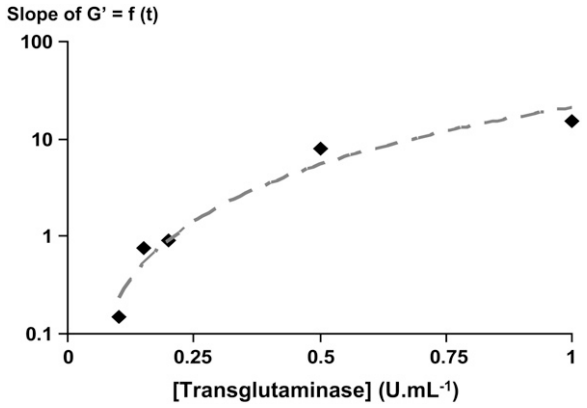

FIGURE 3 Evolution of the enzymatic reaction considered through the physicochemical properties (G′ evolution) of the gel as a function of transglutaminase concentration measured 1 h after the gel point at 40°C: $y = 15x^{1.85}$, $R^2 = 0.98$.

containing transglutaminase in the same conditions (physicochemical gel). As previously described, the helix ratio depends on the internal network of the gel (19), but triple helices are not thermally stabilized by covalent bonds as they melt at the same temperature as those constituting the physical network.

Designating g, s, and f as the time-dependent concentrations of bound gelatin chains, soluble proteins, and their corresponding fragments, respectively, T, the transglutaminase concentration, and P, the protease concentration, we obtain the set of three ordinary differential equations:

$$\frac{dg}{dt} = -\frac{k_P P}{K_P + g} g + \frac{k_T T^{\beta}}{K_T + s} s(+ V_H)$$

$$\frac{ds}{dt} = \frac{k_P P}{K_P + g} g - \frac{k_T T^{\beta}}{K_T + s} s - \frac{k'_P P}{K_P + s} s(-V_H)$$

$$\frac{df}{dt} = \frac{k'_P P}{K_P + s} s$$

$$g_t + s_t + f_t = s_0.$$

Protease autolysis is negligible as $(dP/dt) = -(k_P P^2)/(K_P + P)$ has a $V_{max} = 2.5 \times 10^{-7}$ min^{-1} with $P = 16 \times 10^{-3}$ M.

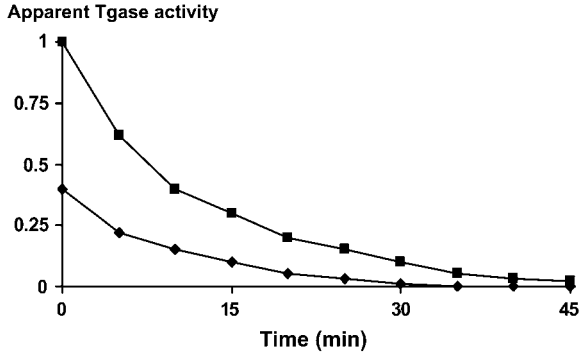

FIGURE 4 Example of evolution of enzyme activity in the gel for 1 unit mL^{-1} (■) and 0.4 units/mL^{-1} (♦) transglutaminase.

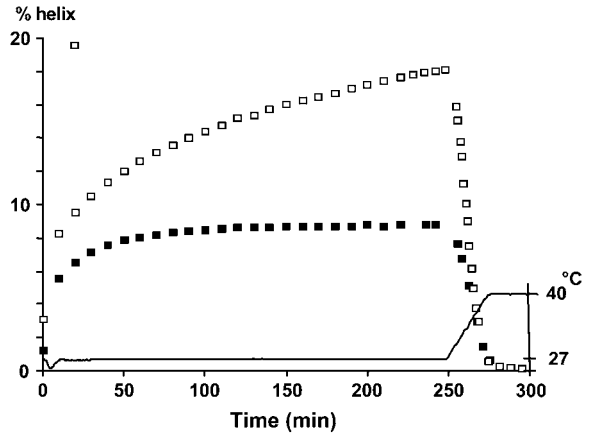

FIGURE 5 Triple helix evolution as a function of time and temperature for a physical gel (□) or a physicochemical gel (■).

Parameter values

Average values from the literature and our own previous experiments were chosen as follows. For kinetic constants, $K_P = 1$ mM; $K_T = 8$ mM; $k_P = 1000$ min^{-1}; $k_T = 100$ min^{-1}.

The gelatin concentration is 50 g/L^{-1}, which is equivalent to 0.45 M of peptide bonds, assuming an average molecular weight of 110 per amino acid residue.

With respect to thermolysin specificity (23), only 15% of gelatin peptide bonds are recognized by the enzyme. The substrate concentration for thermolysin is thus 68 mM, equivalent to 8.5 K_P, which corresponds to 90% saturation. Furthermore, gelatin contains only 4% lysine (average value for various sources) hence, S_0, the concentration for transglutaminase is 17 mM, corresponding to 2 K_T (66% of saturating concentration). As an interconverting cycle is implemented, substrate concentration can be considered nonlimiting for enzyme reactions, at least until a gel is obtained.

Possible side reactions were evaluated. Transglutaminase is a potential substrate for metalloproteases (24). Bearing in mind the sequence of the used transglutaminase (25) and its concentration equivalent to 76 nM as a substrate for thermolysin, the probability of hydrolysis of transglutaminase versus gelatin by the protease is only $\sim 10^{-6}$; it is thus neglected in the model. Similarly, the concentration of thermolysin available for transglutaminase reaction is only 0.1 nM, to be compared to 17 mM for gelatin; the cross-link of thermolysin by transglutaminase is not considered in the model.

For V_H calculations, the fact that three gelatin chains are needed to form a triple helix was taken into account.

Theoretical results

From the set of values described above, we calculated the evolution of the bound gelatin chains (g) as a percentage of the total protein concentration. An example is shown in Fig. 6 for a ratio of transglutaminase/protease activity of 80 at 40°C where no triple helices were formed.

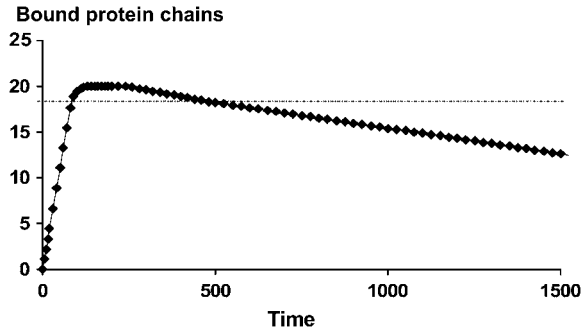

FIGURE 6 Theoretical evolution of gelatin-bound chains (%) as a function of time (adimensional) at 40°C with $k'_P = 0.05\ k_P$ and k_T. $T/k_P\ P = 80$.

Fig. 7 is another example at 27°C where the effect of triple helices added to covalent bonding. It has been previously shown (22) that when transglutaminase has created 18% of the possible N^{ε}-(γ-glutamyl)-L-lysine bonds in gelatin, a gel was generated. We thus consider having formed a gel from this value of bound gelatin chains.

These theoretical results show that, as we assumed, the rate of bound protein chains increases over the threshold needed for a sol/gel transition and after a while decreases spontaneously so that a solution should be obtained again. The kinetics as well as the overall properties of the gels depend on the relative enzyme activities and on operational conditions. For example, at 27°C, gelation should occur faster and more chains should be bound than at higher temperatures so that more stable gels would be observed; the double transition also needs less transglutaminase to occur in this case. From this model, we determined enzyme activity ratios where the double transition could occur.

Experimental observations

Gels were then realized in the conditions defined through the theoretical model. In the given example, the two enzyme reactions were carried out at 40°C, a temperature where no physical gelatin gel can be obtained, so that only transglutaminase-catalyzed covalent bonds are responsible for the gelation process. The properties of the gel were followed by rheology, as previously described (19). Under these conditions, a gel is obtained when the storage modulus G' becomes higher than the loss modulus G'', that is to say when $G'/G'' > 1$. At constant gelatin concentration (50 g/L^{-1}), various transglutaminase/protease ratios (T/P) were tested and also the enzyme activity was varied for a constant T/P ratio. Fig. 8 illustrates a typical result obtained under these conditions for a chemical gel at 40°C. The analysis of the results indicates that the gelation process is an accurate phenomenon under these conditions. With a T/P ratio of 75 no gel was obtained (the experiment was carried out over 80 h; data not shown), whereas increasing this value to 80 allowed gelling to proceed. This first phase transition occurs in 19 min, which is slower than for a gel without protease (8.5 min; data not shown). As predicted by the model, the gel is evolving: its viscoelasticity increases to reach a maximal value ($G'/G'' = 26$) in 165 min, then dissolution begins, being achieved in 760 min. The process is irreversible; no spontaneous gelling was observed beyond this point, which is consistent with the hypothesis of small fragments discussed above in the theoretical model.

The overall behavior of the preparation is also strictly dependent on the enzyme activity. A slight decrease in the enzyme concentrations (0.4 unit instead of 1 unit transglutaminase, T/P ratio constant) results in an increase in the gelling time (56 min instead of 19 min), higher viscoelastic properties ($G'/G'' = 71$ after 10 h), and a longer gel phase (solubilization needs 80 h).

Varying temperature, completely different properties were observed (Fig. 9) as previously suggested from the theoretical model. At a temperature where both triple helices and

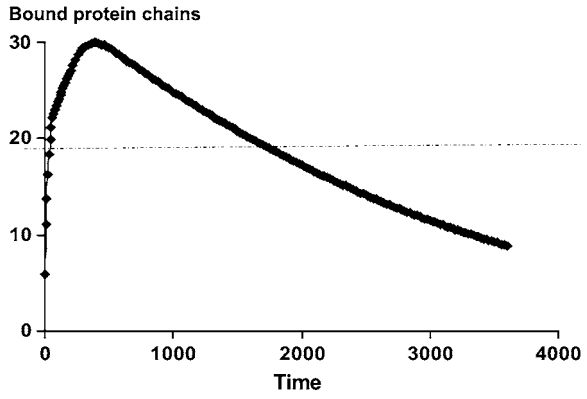

FIGURE 7 Theoretical evolution of gelatin-bound chains (%) as a function of time (adimensional) at 27°C with $k'_P = 0.05\ k_P$ and k_T. $T/k_P\ P = 10$.

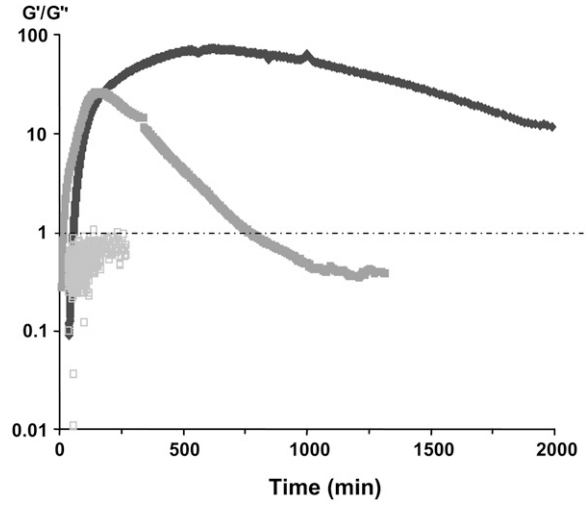

FIGURE 8 Evolution of the viscoelastic properties of ephemeral gels as a function of time (minutes) at 40°C for $T/P = 80$ with $T = 0.4$ units/mL^{-1} (♦) or $T = 1$ units/mL^{-1} (*gray*) and for $T/P = 75$ with $T = 1$ units/mL^{-1} (□). A gel phase is obtained when G′/G″ > 1.

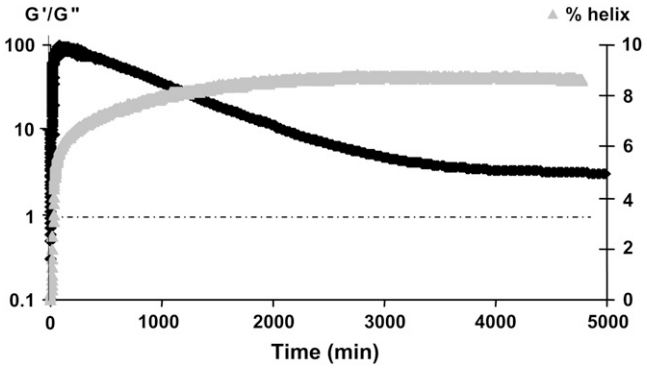

FIGURE 9 Evolution of the viscoelastic properties and triple helix content of a physicochemical ephemeral gel as a function of time at 27°C for $T/P = 80$.

covalent bonds contribute to the network, the gel resistance is increased. These results are consistent with the ones previously observed when adding protease outside the gel (19). One may remark that helices are not degraded during the gel/sol transition. This is due to the particular choice of the protease, thermolysin, which preferentially recognizes hydrophobic residues with large lateral chains (23); as they are rare in helices, thermolysin should preferentially act on the random coil part of the gel chains as previously shown (19). This illustrates the main difference between gelatin consisting of local triple helix association linked by random coils and collagen whose triple helices regulate metalloprotease activities (26).

This special recognition of gelatin by thermolysin was confirmed as just after the gel/sol transition had occurred, a second sol/gel transition has been generated in some cases by decreasing the temperature. The gel formation is then due to the additional appearance of triple helices, which are insensitive to thermolysin. However, such a behavior cannot be generalized, as when the hydrolysis reaction has gone far helices are formed but no gel phase is obtained when decreasing temperature. In this case, gel solubilization is irreversible.

These results illustrate that varying the temperature we were able to generate ephemeral gels with various lifetimes and mechanistic properties. A full range of controlled properties can be obtained by modifying the protein nature and concentration, the protease specificity, the ratio of the two antagonistic enzymes, and the activity of each enzyme. The details will be described elsewhere.

DISCUSSION

A large amount of work has been dedicated in the past years to the catabolic action of proteases. Our main objective in this article consisted of the concomitant introduction of transglutaminases: as these wide-spread enzymes can turn soluble proteins into insoluble lattices, their influence on the mechanical properties of biological gels appears to be the reverse of the proteases' action. We thus treated proteases and transglutaminases as reverse catalysts of a futile cycle in which proteins and their soluble proteolysis fragments are interconverted. In vivo, however, few enzyme categories are known to form bienzymatic cycles. The main but ubiquitous example concerns enzymes involved in phosphorylation: dephosphorylation events. Thus most of the previously published studies of bienzymatic cycles relate to kinase/phosphatase cycles. Seen from this angle, our model develops a new category of enzyme families possibly involved in a bienzymatic cycle: the protease/transglutaminase activities. In a previous model (13), we described in detail a protease/transglutaminase futile cycle in the case of extracellular matrix remodeling. In this case, the model incorporated ECM production by the cells at a constant rate, r_{im}, so that the (protein + peptide) concentration was not constant. In this first theoretical study, the introduction of new proteins due to cell response to matrix hydrolysis compensated for the leak due to small fragment synthesis. Moreover, a neosynthesis of protease was considered depending on fragment concentration to describe cryptic activities of the peptides, so that the protease concentration was not constant in this model. This led to periodic solutions, indicating that homeostasis of ECM remodeling resulted from an oscillating mechanism, providing a seemingly constant average level. In the model here presented, the protein and the protease concentrations are constant, so that the protease reaction leading to small peptides induces an unavoidable decrease of the quantity of proteins able to link to form a network. No oscillating behaviors can be predicted in this case.

The original part of the example here described resides in the fact that these two enzymes are considered in their capacity not only to modify macromolecules at a molecular scale but also to act as phase transition catalysts. To take this supramolecular aspect into account, we calculated the diffusional constraints due to the appearance of a gel. This introduction of a diffusion-reaction system in the kinetic model of an enzyme futile cycle is a new concept. This is responsible for the nonlinear behavior of the decrease phase observed in Figs. 6 and 7. Through the theoretical model, we have shown that two antagonistic enzyme activities, inside a mixed network, may generate a dynamic modification of protein network which induces a sol/gel transition followed by a gel/sol transition.

Then experimental confirmation of these theoretical results was achieved. Using both proteases and transglutaminases with a gelatin solution, we obtained a completely new type of material, which we term ''Enzgels''. This is the first report in the literature, to our knowledge, where no modification in temperature or medium composition is required to dissolve a gel in vitro. The Enzgels are dynamic protein solutions able to spontaneously cycle from a sol/gel to a gel/sol transition. The interest of this type of material resides in their ability to be completely preprogrammed. The transition depends on the conformation of proteins constituting the network, but the type of bonds forming the network itself is even more important. To better evaluate the

relation between the gel network, enzymatic proteolysis, and mechanical properties, studies with various proteases showing different specificities, activities, and mechanisms are currently being carried out.

These studies also contribute to the knowledge of enzyme behavior in nonconventional media. To our knowledge, the model described here is original in enzymology as it combines a futile cycle with nonenzymatic, nonlinear reactions and takes diffusion-reaction into account. The consideration of macroscopic properties of the medium on biological behavior has been poorly studied as compared to reactions at the molecular scales. However, it relies on a growing amount of reports that the mechanical and physical characteristics of the ECM, such as rigidity or elasticity, can by themselves regulate cell physiology, irrespective of the chemical identity of the proteins the ECM contains (27–29). Through measurements of macroscopic properties of the gels, we were able to evaluate diffusion constraints encountered by enzymes when acting inside the gel. The nonlinear character of these limitations may be of interest in understanding the relation between global gel phase properties and their dynamics. The fact that diffusional constraints are not progressive but occur nonlinearly after the gel point shows that the dynamics encountered here consists of switches between these two states. This observation is in good agreement with sol/gel transition theory, which predicts power laws for the macroscopic characteristics of the gel (14). We had already shown that gel/sol transition could describe the proteolysis of protein gels (3) and that enzyme-catalyzed gel proteolysis is diffusion controlled when a small amount of protease is used (30).

The dynamics of our in vitro system consists of switches between two physical states. This work may contribute to the understanding of complex biological processes such as the matrix remodeling involved in fibrosis or cancer metastasis. Under physiological conditions, the ECM is a physical barrier to cell displacement. To disseminate and form distant metastases, invasive cells counteract this barrier by the production of extracellular proteases that locally degrade the ECM and render it porous to the cells (5,12). It is also known that normal cells react to the solubilization of their ECM by an overproduction of transglutaminase (24,31). Extrapolating our results, we can assume that the switch between two macroscopic states of ECM would be effective only if protease concentration gets higher than a threshold that depends on the cell and its particular ECM gel properties. Additionally, in our model, a parameter zone can be observed where the high transglutaminase activity compensates for the high protease level, allowing coexistence of a gel phase with high protease concentrations. This could be a picture of the situation encountered during cell invasion.

Our mathematical model provides a good tool for determining experimental parameters of bienzymatic cycles leading to phase transitions. It can be extended to other gelling macromolecules, and we are currently working on polysaccharides and their transforming enzymes.

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