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Stenberg, Pål; Roth, Bodil

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ZINC IS THE MODULATOR OF THE CALCIUM-DEPENDENT ACTIVATION OF POST-TRANSLATIONALLY ACTING THIOL-ENZYMES IN AUTOIMMUNE DISEASES

Pål Stenberg[1*], Associate Professor, Bodil Roth[2], Dr Med sci

[1]Lund University, Department of Clinical Sciences Malmö, Clinical Coagulation Research Unit, Skåne University Hospital, S-205 02 Malmö, SWEDEN

[2]Lund University, Department of Clinical Sciences Malmö, Internal Medicine, Skåne University Hospital, S-205 02 Malmö, SWEDEN

*Corresponding author: Tel.: +46 70 96 29 325

E-mail address: pal.stenberg@comhem.se

Postal address: Stenberg, Regementsgatan 11, S-217 53 Malmö, Sweden

Abstract

Post-translational modifications of proteins can generate antigenic conformations that may cause autoimmune diseases in persons with specific HLA-haplotypes. Monocytes and macrophages, attracted to an inflamed site, can release post-translationally acting enzymes, such as transglutaminases and peptidylarginine deiminases. In vivo, the activation of these enzymes is crucial for the further course of event. Our hypothesis is that zinc modulates the activation of these calcium-dependent thiol-enzymes.

Persons with celiac disease carry antibodies against deamidated dietary gluten and against transglutaminase type 2. Similarly, antibodies against citrulline-containing peptides and against peptidylarginine deiminase are detected in patients with rheumatoid arthritis. Thus, in two major autoimmune diseases, antibodies are detected against post-translationally modified proteins and against the thiol-enzymes responsible for catalyzing the modifications.

In vitro, physiological concentrations of zinc reversibly inhibit the calcium-dependent activation of transglutaminases. Zinc attenuates the calcium-induced increase in affinity between transglutaminase 2 and serum from patients with celiac disease. Peptidylarginine deiminases are also inhibited by zinc. Moreover, zinc is rapidly redistributed in animals when an infection is induced.

This pathway starting with an unspecific inflammation and ending up with an immune reaction against a specific tissue constitutes a theme with variations in other autoimmune diseases, such as dermatitis herpetiformis, multiple sclerosis, and type 1 diabetes.

Inhibitors against transglutaminases and peptidylarginine deiminases have a great pharmacological potential. Interestingly, a large portion of the population may have been exposed to such an inhibitor. The primary metabolite of ethanol, acetaldehyde, can probably function as an irreversible inhibitor of these enzymes by forming a hemithioacetal with the thiol group of the active site. Not surprisingly, epidemiological studies have shown that alcohol is beneficial in rheumatoid arthritis. We predict that a similar situation will be observed in multiple sclerosis.

The affinity of chelators such as EDTA and EGTA for Zn^{2+} is three orders of magnitude greater than that for $Ca^{2+.}$ This frequently overlooked complication imposes problems in biomedical research since a restoration of the zinc level can never be achieved in a blood sample which has been anti-coagulated by calcium chelators. The new synthetic direct thrombin inhibitors may offer a better way of preventing coagulation in vitro.

Conclusions: Post-translational modifications are of potential interest in autoimmune diseases. The in vivo activation of calcium-dependent thiol-enzymes catalyzing these alterations, such as the transglutaminases and the peptidylarginine deiminases, is crucial for this pathway. According to our hypothesis, zinc is the modulator of this key function.

INTRODUCTION

With an overall prevalence of 5-8 %, the autoimmune diseases cause a detrimental effect on public health. Since there is no cure, duration is chronic in most cases. Genetic, lifestyle and environmental factors are believed to contribute to the pathogeneses. In order to understand the basic mechanisms, gene mapping has dominated during the last decade. Besides HLA-characteristics, the results in general have not led to greater understanding of the immunopathogenic processes. Epigenetics as well as post-translational modifications might explain part of this disappointment. Instead, a milestone in the understanding of the pathogenesis of a T cell mediated disorder, celiac disease (CD), was established when Dietrich et al in 1997 (1), using a proteomic approach, showed that the antigen of a common biomarker in CD, endomysial antibodies (EMA), is a post-translationally acting thiol-enzyme, transglutaminase type 2 (TG2). Subsequent studies by several groups (2, 3) have explored the fascinating consequences of this major discovery. Indeed, CD has become a model for understanding the basic mechanisms in autoimmune diseases. With the finding of the presence of antibodies against citrullinated peptides (ACPA) in rheumatoid arthritis (RA) (4, 5) involving another posttranslationally acting thiol-enzyme, peptidylarginine deiminase (PAD), the pathogenetic similarities between CD and RA became striking (6).

The enzymes

The mammalian TG-family comprises eight members with potential transamidating activity including the thrombin-dependent plasma Factor XIII (FXIII). They are all calcium-dependent with an essential cysteine residue in the active site and catalyze the intermolecular formation of ε-(γ-glutamyl)lysine pseudo-peptides between specific lysine and glutamine residues. The most well-known physiological function is the FXIII-catalyzed stabilization of fibrin in the terminal stage of blood coagulation (7). The intermediary complex in TG-

catalyzed reactions is a thioester formed between the active site cysteine and a glutamine residue of the substrate with ammonia as the leaving group. In the absence of a lysine group, water can serve as the second substrate resulting in a deamidation of the glutamine residue (Figure 1). In transamidation, acylation is the rate-limiting step (8). On the contrary, the second step is rate-limiting in hydrolysis (9), meaning that the intermediary thioester will be comparatively long-lived.

PADs catalyze the conversion of a positively charged guanidinium group of an arginine side chain into a neutral ureido group (Figure 2). The interest in PADs was piqued when ACPAs were detected in cases of RA.

There are striking similarities between the PADs and the TGs (6). From an autoimmune point of view, antibodies are found not only against their modified substrates but also against the enzymes catalyzing the modifications. Moreover, different isoforms are expressed in monocytes (PAD4 and FXIII) and macrophages (PAD2 and TG2). PADs and TGs have been implicated in a number of physiological and pathological mechanisms, such as induction of apoptosis, cell adhesion, receptor-mediated endocytosis, cell growth and differentiation, modeling of the extracellular matrix, cancer metastasis, CD and RA (10-12).

Zinc

Next to iron, zinc is the most abundant transition metal in the human body. Besides other functions, more than 300 enzymes are zinc dependent. In a few cases, zinc can be inhibitory to enzyme activities. In humans, approximately 90 % of the 2-3 grams of body zinc is located to bone and skin. The highest concentrations of zinc in man are found in retina, the prostate gland, semen and the insulin-producing pancreatic β-cells. Zinc deficiency has a major impact on human health and disease (13).

THE HYPOTHESIS

TG2 is easily activated in vitro by calcium. In vivo, the situation is more complex as illustrated by Siegel et al (14). In general, the intracellular concentration of calcium is too low for significant activity. Moreover, GTP might interfere with the activation. Surprisingly, the majority of extracellular TG2 is also inactive under normal physiological conditions with seemingly sufficient calcium. However, in wound healing models in rodents, TG2 is transiently activated upon tissue injury. Thus, it appears that an important factor is missing in the description of the activation. Based on the following facts, o*ur hypothesis is that zinc is this modulator*:

- In vitro, physiological concentrations of zinc is a reversible and competitive inhibitor of the calcium-induced activation of TG2 and of thrombin-activated FXIII (15)
- Zinc attenuates the calcium-induced increase in affinity between TG2 and CD antibodies (16)
- Zinc is rapidly redistributed in animals during experimental conditions when an infection is induced (17)
- There are several zinc-transporting proteins in man, and the tissue concentration of zinc is not necessarily in equilibrium with the blood concentration
- PAD is also inhibited by zinc but details have not been described (18)

A PUTATIVE PATHWAY FOR THE INITIATION OF AUTOIMMUNE DISEASES

Based on present knowledge in the pathogeneses of CD, RA, dermatitis herpetiformis (DH), multiple sclerosis (MS) and type 1 diabetes (T1D) (see Table 1), we present in this paper a putative common mechanism for the initiation of these autoimmune diseases with zinc as

the major actor. Hypothetically, the primary immunization takes place as illustrated in Figure 3.

The clinical manifestation may start several years after the primary immunization and can be outlined as follows:

- An increased expression of enzymes occurs in the tissue that will be the target for the specific immune reaction. This increase may be initiated by a change of sexual hormones. The increased enzyme activity results in more modified proteins.
- The modified proteins activate the immune system in previously immunized individuals
- Monocytes and macrophages are attracted to the inflamed site and a vicious circle is induced leading to chronicity

This hypothesis, starting with an unspecific inflammation and ending up with an immune reaction against a specific tissue, constitutes a theme with variations.

Coeliac disease

CD is a T cell-mediated reaction against dietary gluten. Although adult cases are identified, CD is mainly diagnosed during childhood. The primary inflammation is probably caused by an intestinal viral infection. The induced immunological reaction causes villous atrophy and subsequent malabsorption. Previously we have postulated the mechanism as illustrated in Figure 4 (2).

Antibodies are formed against deamidated dietary gliadins, but also against TG2. Thus, in CD antibodies are detected against a modified external substrate and against an endogenous enzyme catalyzing this modification. Based on early studies on the kinetics of transglutaminase catalyzed transamidation and hydrolysis (8, 9), we have concluded that the intermediate between TG2 and partly deamidated gliadins is fairly long-lived and might constitute the autoantigen.

Serum zinc concentration is decreased in untreated CD children but normalizes on a gluten-free diet (19).

Dermatitis herpetiformis

DH is probably caused by a mechanism similar to the one in CD. The major difference is that TG3 (epidermal TG), is involved (20). Zinc concentrations in epidermis, papillary dermis and serum in patients with DH are decreased. The mean serum zinc concentration is significantly decreased only in men with DH. There is no correlation between the concentration of zinc in epidermis or dermis and that in serum (21).

Rheumatoid arthritis

Two forms of RA have been postulated, one with and one without ACPA (22). Our hypothesis is limited to the more prevalent form, ACPA-positive RA. Since cigarette smoking dramatically increases the risk, the respiratory tract is considered as the major site for the primary inflammation. The attracted monocytes/macrophages then release enzymes such as PADs and TGs. These are activated by calcium due to a reduced zinc concentration. The result will be citrullinated proteins which will be recognized as antigens by individuals with the specific haplotype HLA-DRB1, also called the shared epitope. A number of citrullinated proteins have been identified in the inflamed joint. Interestingly, in about half of the cases

with ACPA-positive RA antibodies have been detected against PAD, as well (6). Thus, similar to the situation in CD, antibodies against a modified substrate and against the enzyme catalyzing the modification are seen in RA.

Intake of alcohol reduces the risk of ACPA-positive RA (23). The primary metabolite of ethanol, acetaldehyde, may react with nucleophiles such as activated PAD, forming a hemi-thioacetal and inactivating the enzyme. Programs for the development of specific PAD inhibitors are in progress (24).

As expected, FXIII and TG2 originating from monocytes and macrophages, respectively, are present in the synovial fluid of the inflamed joint (6), but their role, if any, in the pathogenesis of ACPA-positive RA is not clear-cut. Dzhambazov et al (25) have shown that TG2 increases the incidence, severity and histopathological manifestations in collagen II-induced arthritis in mice. TG2 and to some extent FXIII are also localized to the extracellular matrix of human normal and osteoarthritic articular cartilage (26).

Low titers of anti-TG2 in RA have been reported by Picarelli et al (27) and by Roth et al (28) while Riente et al (29) did not find elevated levels of anti-TG2. Thus, from an immunological point of view, the role of TGs in RA is unclear. However, TG2 and possibly also FXIII are linked to the formation of specialized invading cell structures, invadopodia, which can break down cartilage in arthritis (30).

Intra-articular injections of cortison reduce the amount of citrullinated proteins in the inflamed joint. A similar injection of low dose zinc acetate should be tried experimentally in order to test our hypothesis that zinc would relieve inflammation by preventing activation of both PADs and TGs.

Multiple sclerosis

In MS, the demyelination of the protecting myelin sheath in CNS is linked to citrullination of several proteins such as the arginine-rich myelin basic protein (MBP). MS is considered to be an autoimmune disease. Like the situation in RA, tobacco smoking is associated with MS. Moreover, deficiency of vitamin D and infection with Epstein-Barr virus have been recognized as contributing factors (31).

Previously, we have hypothesized that an infection in the CNS attracts monocytes and macrophages containing PADs (32). The enzymes are released and activated due to reduced zinc levels. As a result of the citrullination, the basic character of the MBPs is reduced and the neurites are damaged. The modified proteins will then function as antigens in persons with a specific HLA haplotype. However, our screening for ACPAs in MS was negative. We used a commercial kit containing cyclic citrullinated peptides (CCP) as antigens. Nor could we detect antibodies against rabbit muscle PAD. Other forms of citrullinated proteins and of PAD might have given another result. Moreover, future studies of this type should include CSF from MS patients.

As mentioned previously, the intake of alcohol has a protective and health promoting effect in ACPA-positive RA. A way to find out if citrullination contributes to the pathogenesis of MS would be to study the association between MS and the intake of alcohol. To our knowledge, no such studies have been published. We predict that a beneficial effect of ethanol will be observed in MS.

Experimental autoimmune encephalomyelitis (EAE) in mice is also associated with increased levels of citrullinated CNS-proteins (33-35). Interestingly, in PAD2 knockout mice the levels of citrullination is reduced without affecting the induction of EAE (33). On the other hand, a

low-molecular weight PAD inhibitor dramatically attenuates disease in EAE (35). Another way to study the possible involvement of PADs in EAE would be to supply the mice with diluted ethanol instead of pure drinking water. Such a study has been performed in a mouse model for collagen induced arthritis (36).

Similar to the situation in RA, TGs may also be involved in MS. When TG2 knock-out mice were used in EAE, the mice showed decreased disease severity as compared to wild-type mice. Moreover, treatment with a low-molecular weight TG-inhibitor ameliorated disease severity in wild-type mice (37). In man, the presence of TG2 has been demonstrated in astrocytes in MS lesions (38).

Type 1 diabetes

Recently, Chimienti (39) has reviewed the interesting relationship between zinc and diabetes. Hypozincaemia is common in T1D and zinc supplementation attenuates the disease. A zinc transporter (ZnT8) is expressed mainly in the pancreatic islets and facilitates the efflux of zinc from the β-cell.

Autoantibodies to pancreatic β-cell antigens are important serological markers of T1D. The antigens recognised during the 1980/90´s are insulin, glutamic acid decarboxylase (GAD65 kDa isoform) and IA-2, another islet cell antigen. In 2007, Wenzlau et al (40) showed that ZnT8 is a major autoantigen in T1D. The region around position 325 seems to define the major epitopes (41). At this position there are three isoforms of ZnT8 containing glutamine, tryptophan or arginine (Q; W; R, respectively) with somewhat different antigenic specificities in childhood T1D.

Recently, post-translational modifications of proteins in T1D have attracted interest (42). We would like to add the possibility that ZnT8Q and ZnT8R are subjected to post-translational

modifications catalyzed by TGs and PADs, respectively. Such modifications may affect the zinc transporting ability and cause a viscous circle. Moreover, in vitro experiments with ELISA or RIA should include addition of zinc to the ZnT8 protein in order to mimic in vivo conditions.

OTHER IMPLICATIONS

The affinity of chelators such as citrate, EDTA and EGTA for Zn^{2+} is significantly greater than that for Ca^{2+}. This difference imposes theoretical and practical problems in biomedical research. Obviously, a restoration of physiological levels of zinc can never be done in a blood sample which has been anti-coagulated by calcium chelators. Thus, if zinc has a role in the mechanism being studied, the result may turn out as ambiguous. Interestingly, it has been suggested that zinc is an important mediator of haemostasis and thrombosis (43). The new synthetic water-soluble direct thrombin inhibitors, such as melagatran, could possibly offer a better way of preventing coagulation in vitro.

Moreover, our experience indicates that dithiothreitol (DTT) is a potent chelator of zinc. DTT is commonly used as an anti-oxidizing agent in experiments with thiol-enzymes.

Furthermore, calcium dependent thiol-enzymes isolated from zinc-rich biological material such as the prostate gland or the retina may not be activated by the addition of calcium unless a zinc-chelator has been added. On the other hand, a protein such as ZnT8 produced in vitro by transcription/translation may not possess the biologically active conformation unless zinc has been added.

CONCLUSIONS

Post-translational modifications are of potential interest in autoimmune diseases. The in vivo activation of calcium-dependent thiol-enzymes catalyzing these alterations, such as the TGs and the PADs, is crucial for this pathway. According to our hypothesis, zinc is the modulator of this key function.

In order to approach the challenging task of understanding the pathogeneses, autoimmune diseases should be studied and analyzed collectively as well as individually.

Conflict of interest statement

We hereby certify that there are no financial or other relationships that might lead to a conflict of interest. The manuscript has been read and approved by both authors.

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Table 1. Putative enzymes, antigens and associated HLA in the five autoimmune diseases

Disease	Enzyme	HLA	Antigens
CD	TG2	DQ2/DQ8	Deamidated gliadin, TG2
DH	TG3	DQ2/DQ8	Deamidated gliadin, TG3
RA (ACPA positive)	PAD, TG2, FXIII	DRB1	ACPAs, PAD
T1D	TGs, PADs	DQ8/DQ2	GAD 65, IL-2, insulin, ZnT8
MS	TGs, PADs	DRB1	Myelin basic proteins (MBPs)

Captions to figures

Figure 1: Transglutaminase catalyzed reactions.

The enzyme is activated by calcium. Zinc counteracts the activation. The exposed nucleophilic thiol of the active site then forms an intermediary thioester with a peptide-bound glutamine residue and ammonia is released. In the presence of a protein-bound lysine residue, transamidation takes place forming intermolecular pseudo-peptide bonds. In the absence of a primary amine or at a site with low pH, water will serve as the second substrate resulting in deamidation.

Figure 2: Peptidylarginine deiminase catalyzed citrullination.

PAD is activated by calcium. Zinc counteracts this process. The positively charged side chain of a protein-bound arginine residue is then converted to a neutral ureido group. Ammonia is formed during the reaction.

Figure 3: A putative pathway for the initiation of autoimmune diseases.

An unspecific inflammation caused e.g. by infections, chemical agents, or physical or mental stress, attracts monocytes and macrophages. The site of the inflammation can be the lungs, the oral cavity, the joints, the skin, the intestinal or urinary tract, the pancreas, CNS, etc and is not restricted to the tissue that later will be the target of the specific immune system.

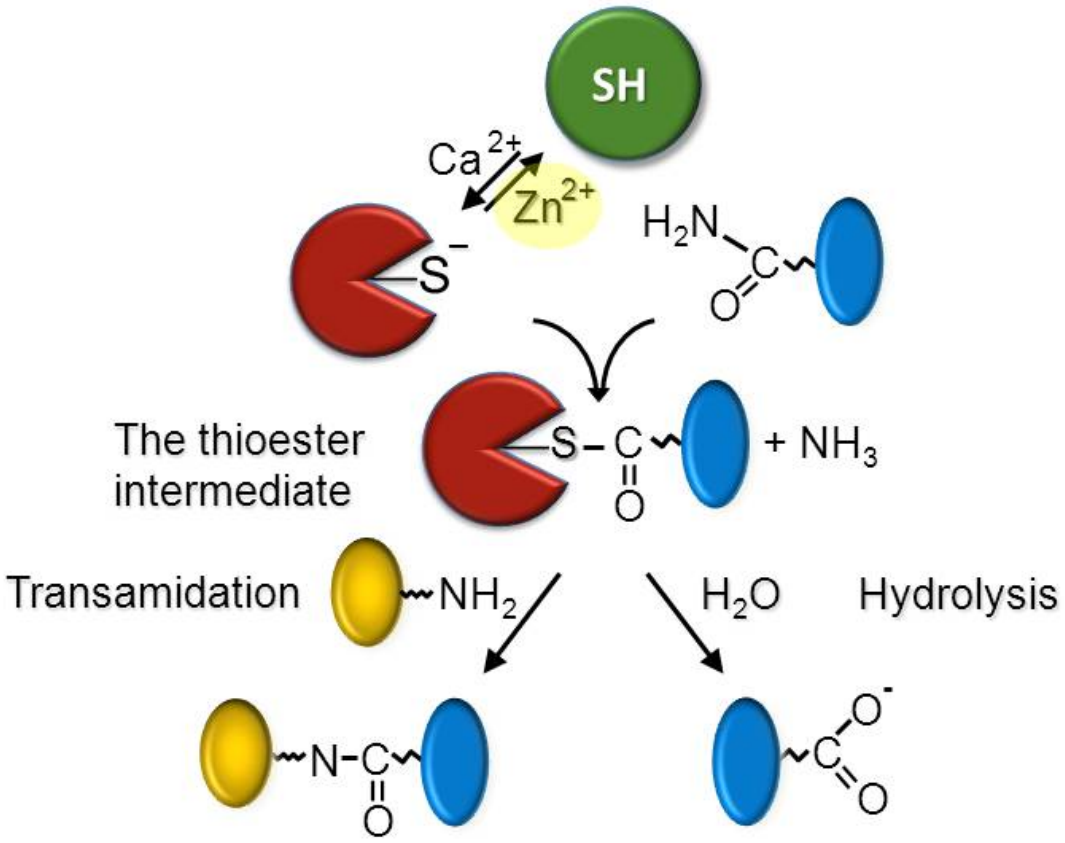
Calcium-dependent posttranslationally acting thiol-enzymes are released from the monocytes/macrophages and activated by calcium due to a reduced concentration of zinc in the inflamed area. The activated enzymes catalyze the modification of proteins. During this

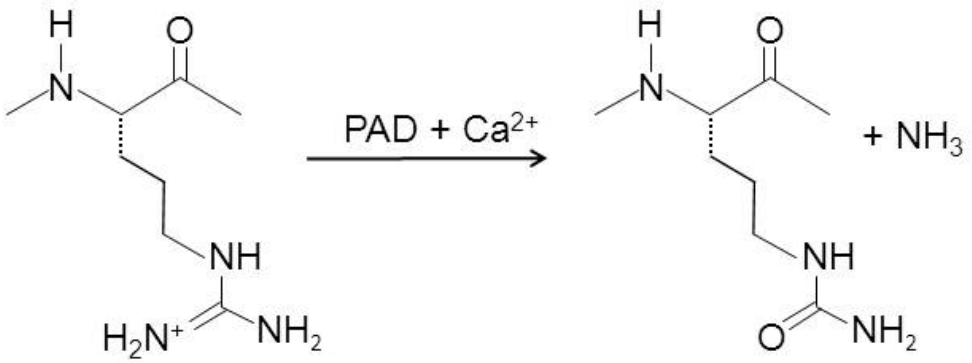
process, an intermediate is formed between the enzyme and the substrate. The altered conformations render the modified proteins immunogenic by allowing attachment of fragments of the enzyme-substrate complex onto specific HLA-molecules and effector T cells are activated. The immune response results in expression of antibodies against the modified proteins.

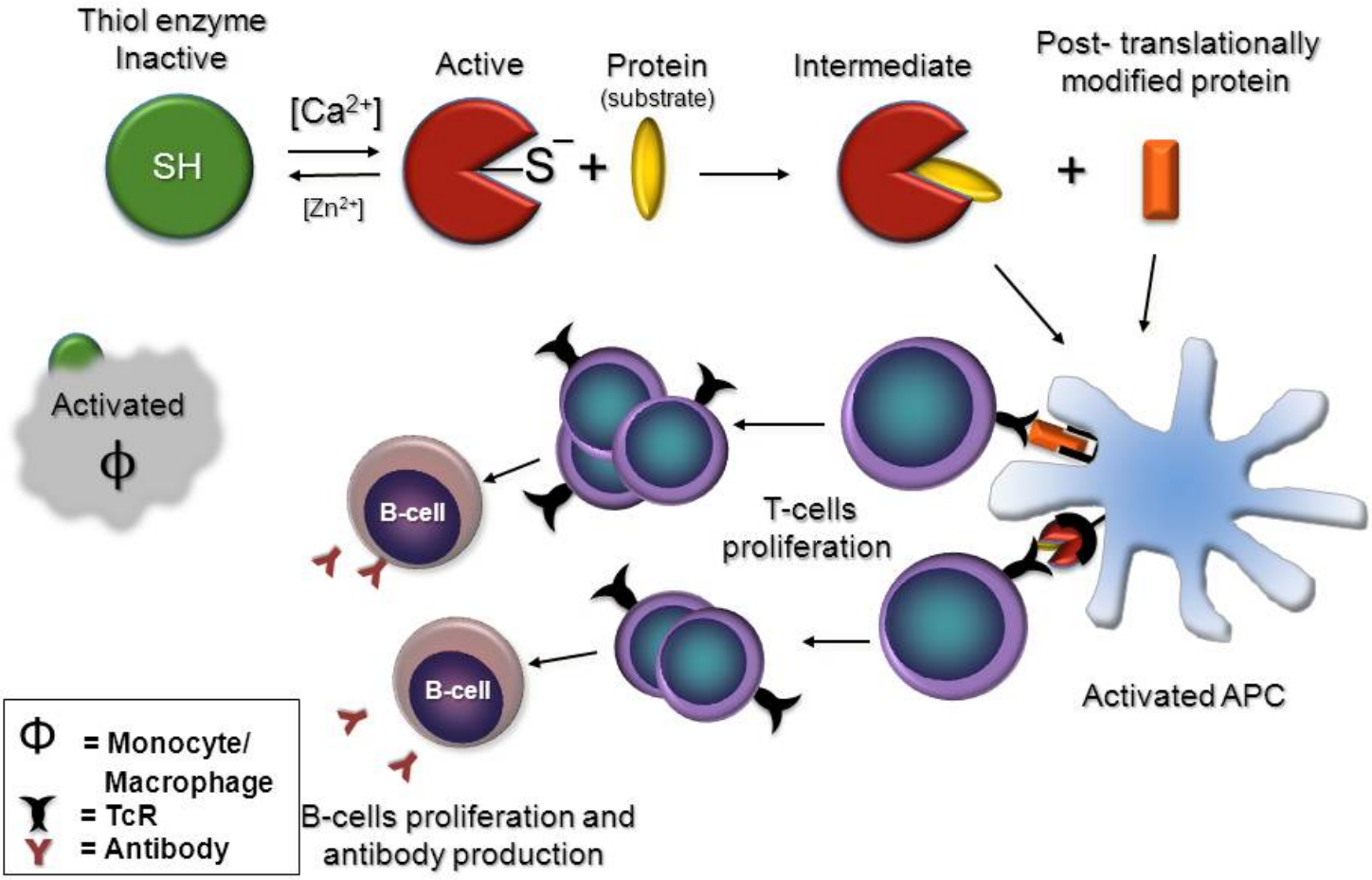
Figure 4: The initial steps in the pathogenesis of celiac disease.

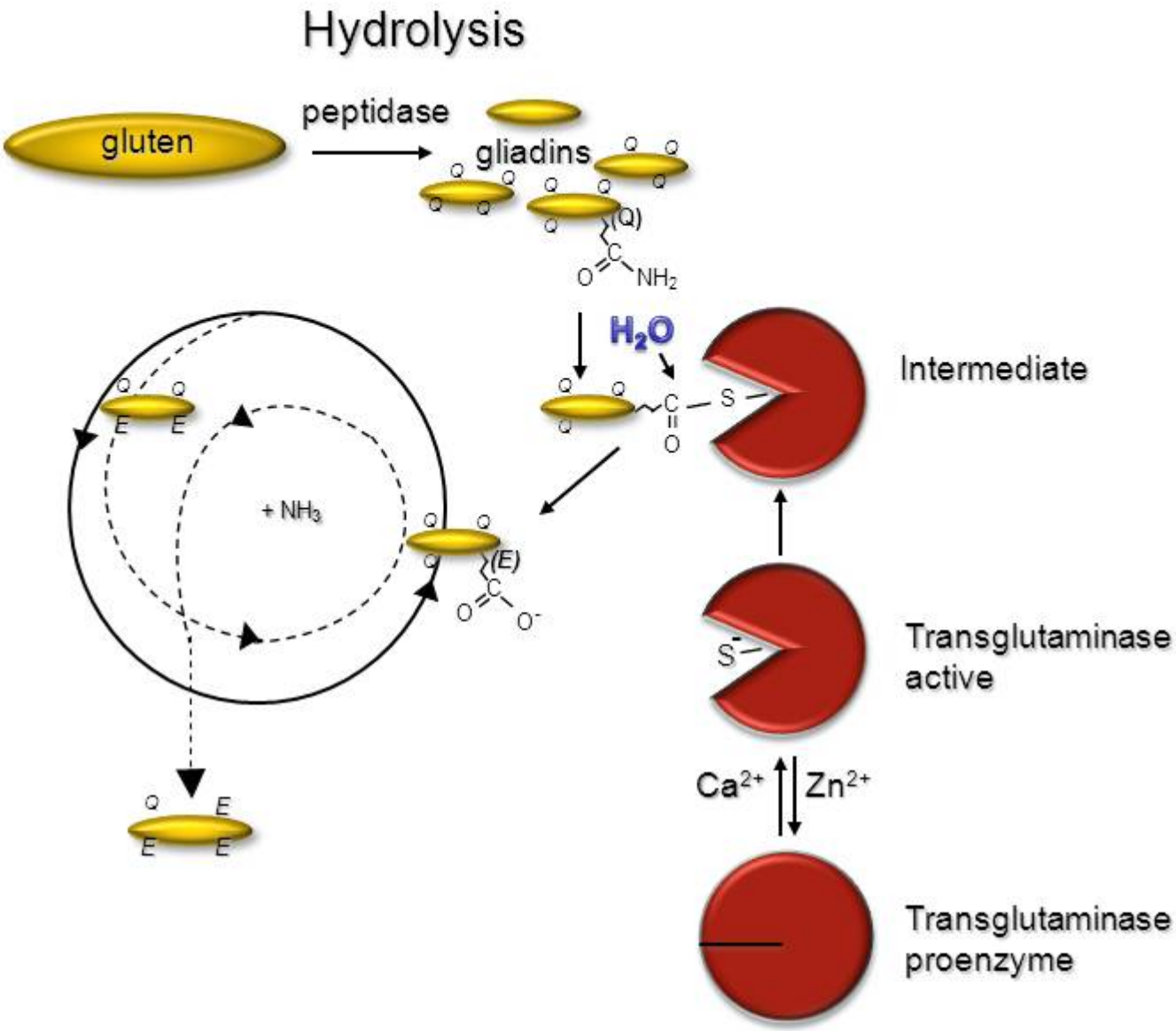
Dietary gluten is digested by peptidases. Due to a subnormal concentration of zinc at an inflamed site of the intestinal tract, TG2 is activated by calcium. In a given order, specific glutamine (Q) residues in the formed gliadins function as substrates for activated TG2. Since there are no lysine residues in gluten, the normal reaction, a transamidation, cannot take place. Instead water function as the second substrate resulting in a deamidation of the glutamine residue, forming a negatively charged glutamate (E). The cycle is then repeated. During this process a thioester intermediate is formed between the active site thiol of TG2 and a glutamine residue of a partly deamidated gliadin peptide. This complex is comparatively long-lived and constitutes the neo-antigen.

Transglutaminase – the principle pathways




H
O
N
PAD + Ca2+
+ NH3
NH
H2N+
NH2
O
Arginine residue
Citrulline residue


Thiol enzyme
Inactive
SH
[Ca2+]
[Zn2+]
Active
S−
Protein
(substrate)
Intermediate
Post- translationally
modified protein
Activated
ϕ
B-cell
T-cells
proliferation
Activated APC
B-cell
Φ = Monocyte/
Macrophage
= TcR
= Antibody
B-cells proliferation and
antibody production


Hydrolysis
gluten
peptidase
gliadins
(Q)
NH2
H2O
Intermediate
S
+ NH3
(E)
Transglutaminase
active
Ca2+
Zn2+
Transglutaminase
proenzyme