

Hypothesis

Accelerated removal of deamidated proteins and endogenous electric fields: possible implications

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Abstract. Changes in protein structure through the spontaneous deamidation of asparaginyl (Asn) and glutaminyl (Gln) residues have been observed in many proteins. Amide residues were supposed to serve as clocks for development and aging. Deamidated proteins are rapidly degraded by as yet unclear molecular mechanisms. Deamidation leads to elevation of the ratio of charged *versus* polar residues (CH-PO) of a protein and to a decrease in its pI value. We had reported that those enzymes, characterized by a high CH-PO, are prone to inactivation and loss of ordered structure by exposure to direct current from low voltage in solution. Nano-local endogenous electric fields arise within cells. Endogenous currents may cause the unfolding of the products of deamidation at Asn. In turn, these unfolded proteins would be removed, likely by proteolysis.

Key words: Aging — Asparagine — CH-PO — Deamidation — Direct electric current — Glutamine — Proteasome

Abbreviations: CH-PO, ratio of charged *versus* polar residues of a protein; dc, direct electric current; Asn, asparagine; Gln, glutamine.

Protein deamidation

Many biological peptides and proteins possess labile asparaginyl (Asn) and glutaminyl (Gln) residues. Deamidation of Asn and Gln residues is now recognized as one of the major pathways of spontaneous protein degradation, together with protein aggregation, oxidation, and glycation (Robinson 2002; Reissner and Aswad 2003; Li et al. 2006).

The first isolation and characterization of the amidated and deamidated forms of a naturally occurring peptide was accomplished by counter-current distribution of insulin in 1952 (reviewed in Robinson 2004a). However, only about 40 years ago, it was discovered that deamidation occurs *in vivo* under genetic control, over a very wide, physiologically relevant deamidation rate range (Robinson et al. 1970). Subsequently, much has been learned, especially about Asn, whose deamidation rate is faster than Gln. Between 1971 and 1973, Robinson and co-workers examined the deamidation

of 65 synthetic peptides labeled with C¹⁴ and measured the deamidation rates at pH 7, directly measuring the sequence-determined deamidation rates of model peptides (reviewed in Robinson 2004a). It is now established that a large percentage of proteins deamidate to a substantial extent during their biological lifetimes.

The deamidation of Gln proceeds both enzymatically and non-enzymatically. By contrast, only the nonenzymatic deamidation of Asn has been reported. Deamidation rates seem to depend upon the sequence and higher-order structure of the protein and to affect protein structure and function in natural and engineered mutant sequences (Clarke 2003). In fact, since the side chains of Asn and Gln are not charged, deamidation introduces a negative charge at the site of occurrence, affecting the structure of the peptides and proteins in which they are incorporated. The occurrence of Asn and Gln in proteins was determined by Robinsons (2004b) from a set of 4835 representative proteins from the January 2003 PDB databank. Authors found that about 72% of proteins possessed more Asn and 63% have more Gln than expected by chance. The research literature on the history deamidation was reviewed and summarized (Robinson 2004a).

Reaction mechanisms of deamidation

The reaction mechanisms of nonenzymatic deamidation have been extensively studied. In eukaryotes, the process of Asn deamidation is non-enzymatic, spontaneously occurring at neutral pH. It proceeds primarily through a succinimide mechanism with also a small amount of direct hydrolysis. To date, enzymes involved in targeted deamidation of Asn in proteins have been only identified in prokaryotes (Horiguchi et al. 1997). Spontaneous Gln deamidation proceeds through a glutarimide, but, since this reaction is relatively slow, direct hydrolysis is more significant. The studies of Robinson et al. (2004a) on synthetic C¹⁴ labeled peptides showed that deamidation rates increased by His and Ser neighboring to Asn with the effect larger for the carboxyl-side residue are affected by steric hindrance from the side chains of the nearest neighboring residues to Asn and Gln and slowed by secondary and tertiary structure (Geiger and Clarke 1987; Robinson and Robinson 2004b). In addition, D-Asn and D-isoaspartyl residues can form (Reissner and Aswad 2003). Under physiological conditions, deamidation of Asn can occur with half-times as short as 6 h (Kim et al. 1999; Robinson and Robinson 2004b). Enzymes have been found that deamidate carboxyl terminal Asn (Kikuchi and Sakaguchi 1972) and amino terminal Asn (Balogh et al. 2000), however, these have no ability to deamidate the side chains of Asn. Asparaginases, acting on free asparagine, are also known.

The enzymatic deamidation of Gln residues by transglutaminases, proteases, and glutaminases, is widely distributed from microorganisms to mammals and is involved in several ordinary and pathological processes. For example, tissue transglutaminase (TG2), a calcium-dependent multifunctional enzyme, produces Gln deamidation as a side reaction that can modify proteins by transamidation or deamidation of specific Gln residues (Lorand 2002). TG2 deamidating activity seems to play a major role in the pathogenesis of celiac disease, as it generates deamidated gliadin peptides that are recognized by CD4⁺ T cells (Mazzeo et al. 2003).

Pivotal role of deamidation in the regulation of biological properties

It seems that the physiological significance of deamidation lays in the alterations of tertiary structure of a protein it produces. For example, deamidation of triose phosphate isomerase (P00939, ExPASy) decreases subunit-subunit interactions, exposing the hydrophobic interface to the aqueous environment (Sun et al. 1995). Deletion experiments showed that deamidation of two Asn of Bcl-x_L disrupt its antiapoptotic activity, as the negatively charged deamida-

tion products alter Bcl-x_L tertiary structure (Deverman et al. 2002). The glial myelin basic protein (MBP) (P02686, ExPASy) undergoes deamidation of Gln producing Glu as well as of Arg, producing citrulline (Coenen et al. 2007), which decreases its cationic properties, in turn affecting adhesion to the two cytosolic layers of myelin sheath (Min et al. 2009). Differential deamidation regulates cellular and developmental processes (Li and Thompson 2002). Moreover, deamidation leads to faster degradation *in vivo* (Robinson and Robinson 2004a). However, the knowledge of critical passages of the removal of the deamidated proteins is still limited.

A computation procedure for quantitatively combined primary-structure determined deamidation rate and the higher order structure modulation of that rate has been developed for 170,014 Asn residues in 13,335 proteins (Robinson 2002; <http://www.deamidation.entrowave.com>). The computed rates show that significant amounts of deamidation may be expected to occur in a substantial percentage of proteins under physiological conditions. It is now established, therefore, that a large percentage of proteins deamidate to a substantial extent during their biological lifetimes. Deamidation can control *in vivo* protein turnover. The protein pool is a dynamic, time-dependent array of protein molecules, whose structures are continuously changing under the control of miniature amide clocks imbedded in each protein (Robinson 2002). The genetically specified primary sequence control of nonenzymatic deamidation of Asn and Gln under physiological conditions provides a range of deamidation half-times between about 1 day and 50 years. The combination of multiple amides in proteins and the modulation of deamidation by secondary, tertiary, and quaternary structure provide a currently known range of physiological protein deamidation half-times between a few hours and more than a century.

Deamidation and aging

The hypothesis that deamidation may serve as a molecular clock in aging of organism was first published by Robinson, McKerrow, and Cary in 1970 (Robinson et al. 1970). Thereafter, this hypothesis became known as the “deamidation theory of aging” and took its place among the many other biochemical processes that have been proposed as the mechanisms of aging (Moffat 1980). Analysis of protein sequence suggests that Asn and Gln ability has been positively selected in the evolution of proteins, possibly playing a role in the regulation of time-dependent biological processes such as protein breakdown, development and aging (Robinson and Robinson 2004a). Also, a correlation was found between the percentage of amide residues, and the

in vivo turnover rate in 12 proteins. Deamidation would be a molecular clock, determining the mean lifetime of certain proteins *in vivo*. If Asn serves as a ubiquitous molecular clock, a lack of natural asparaginases is expected, in fact they have never been described. This fact reflects a difference between Asn and Gln in their usefulness as biomolecular clocks.

The hypothesis that nonenzymatic deamidation serves as an ubiquitous molecular clock for the regulation of biological processes has been strengthened by studies showing that nonenzymatic deamidation controls the *in vivo* turnover rates of cytochrome *c* and aldolase. Deamidation of cytochrome *c* leads to its rapid catabolism *in vivo*, with rates equivalent to the turnover rate (Robinson 2004b). Similarly, the deamidation of the aldolase C-terminal sequence and the *in vivo* turnover proceed at the same rates (Robinson and Robinson 2004b).

CH-PO parameter

A lot of thermostable proteins display a large difference between charged (Asp, Glu, Lys, Arg) *versus* polar (Asn, Gln, Ser, Thr) amino acids (Suhre and Claverie 2003). CH-PO value for hyperthermophilic proteins is higher ($10 \div 15.0$) in comparison with mesophilic ($-5.0 \div 5.0$) ones (Suhre and Claverie 2003). Polar residues, tending to introduce the aqueous solvent into the core of the protein, are less represented in hyperthermophilic proteins, that are stabilized by a network of ion bonds at the protein surface (Kumar and Nussinov 2001). A few mesophyle enzymes still display high CH-PO (e.g. 11.37 for human DNA polymerase catalytic subunit; 25.8 for human DNA topoisomerase), and may be

vestigial of our ancestors dwelling in drastic environmental conditions. On the other hand, other factors contribute toward thermal stability of other thermostable proteins (i.e. increase in secondary structure, hydrophobic interactions, disulphide bonds and oligomerization) (Sakuraba et al. 2003; Yano and Poulos 2003).

Endogenous direct currents

Extra- and intra-cellular electrical currents arise *in vivo*, as a result of the diffusion and convection of charged species, spanning a broad frequency range, from the milliHertz of injury currents up to hundreds of Hertz around excitable membranes (reviewed in McCaig et al. 2005). Currents of $100 \mu\text{A}/\text{cm}^2$ have been measured at the blastopore of *Xenopus* embryos. There is growing evidence that endogenous electric fields (EF), and consequent endogenous dc, are present at wounds and in areas of active cell growth and migration during development. In rat cornea wound healing seems regulated by the wound-induced EF (McCaig et al. 2005). Endogenous EF are essential to regulate cell behaviour during tissue morphogenesis and regeneration (Song et al. 2004; Saunders and McCaig 2005). It would be interesting to know whether there are variations in protein deamidation in the development and wound healing processes. It was reported that the *Escherichia coli* cytotoxic necrotizing factor 1, which catalyzes the deamidation of the Rho GTP-binding proteins at a specific Gln, impairs intestinal epithelial wound healing (Brest et al. 2004). Also, TG2 affects cell-matrix interactions in cell spreading, migration and extracellular matrix reorganization in wound healing (Hasan et al. 2006). However, in the latter two cases deamidation is not spontaneous.

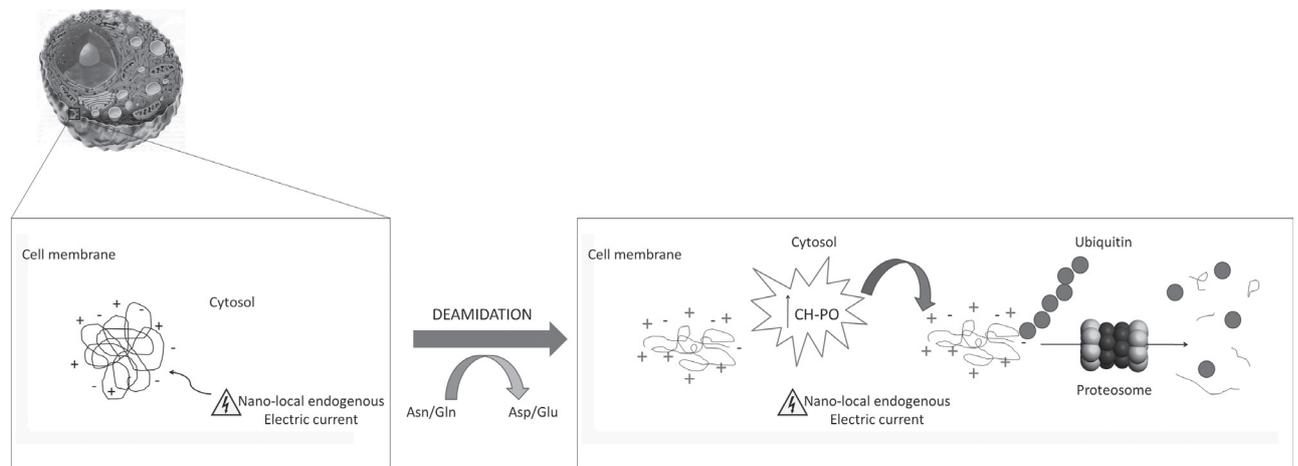


Figure 1. Hypothetic route for the degradation of deamidated proteins. It is supposed that proteins undergoing an increase in CH-PO over a threshold value of (10 ± 1.4) and a decrease in pI upon spontaneous deamidation would be prone to permanent unfolding by nanolocal direct-current electric fields, recognized as damaged and sent to proteasomal degradation.

Table 1. Measured *versus* theoretically calculated *pI* values for 12 proteins in the paper from Dice and Goldberg (1975)

Protein	Swiss Prot	Total AA number	Measured <i>pI</i>	Calculated <i>pI</i>
δ -amino levulinate synthetase	P06214	330	5.9	6.32
Aspartate amino transferase	P00507	430	5.6	9.13
Catalase	P04762	526	6.7	7.07
Ferritin	P19132	181	5.7	5.86
Glucose-6-phosphate dehydrogenase	P05370	514	5.3	5.97
Histidase	P21213	657	5.4	6.07
Ornithine amino transferase	P04182	439	5.4	6.53
Ornithine decarboxylase	P09057	461	4.1	5.21
Phosphoenolpyruvate carboxylase	P07379	622	5.0	6.09
Pyruvate kinase	P11980	530	6.0	6.69
Tryptophan oxygenase	P09810	444	5.0	6.3
Tyrosine amino transferase	P04694	454	4.1	5.27

Swiss Prot, ExPASy proteomics server accession number (www.expasy.org); total AA number, number of aminoacids (AA); measured *pI*, value measured by the author; calculated *pI*, value theoretically calculated according to ExPaSy.

Interaction of dc and proteins with elevated CH-PO

Interaction between electric current and proteins is a little explored topic. We reported that exposure of *Crotalus atrox* venom to dc in solution caused inactivation of venom phospholipase A₂ and metalloproteases (Panfoli et al. 2007). We also reported that enzymes with CH-PO higher than 10.0 are irreversibly inactivated by dc exposure (in the range

0–0.7 mA) from low voltage (0–10 V). By contrast, those with CH-PO in the range –5 and +3 are not (Calzia et al. 2009). Inactivation was dependent on the ionic strength of the medium and not on the net charge of the protein. The effect seemed to be due to the current density. Some of the inactivated enzymes underwent a structural modification measurable by circular dichroic spectroscopy. It was concluded that CH-PO can predict protein inactivation by dc exposure (Calzia et al. 2009). Interestingly, as stated

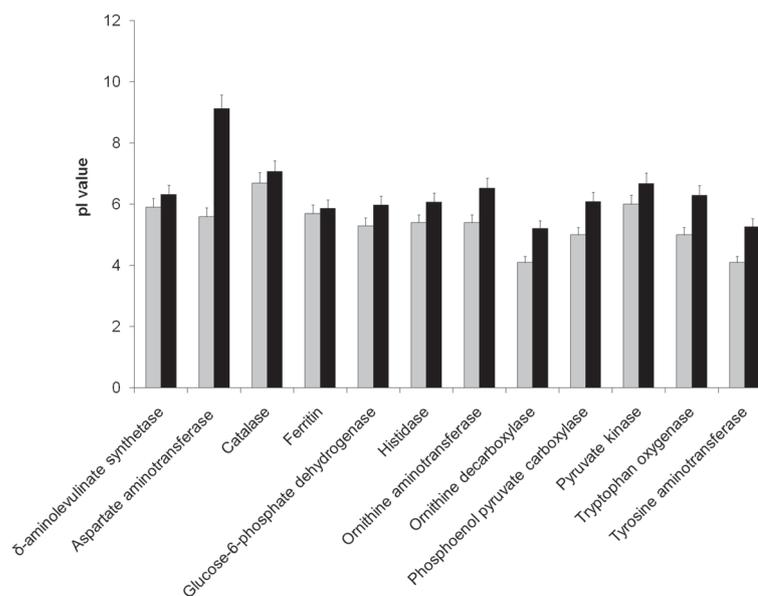


Figure 2. Theoretical *versus* measured *pI* values for 12 proteins selected from Table 1 in the paper Dice and Goldberg (1975). Figure reports *pI* value, as measured by the authors (grey columns), or *pI* value theoretically calculated (black columns) according to ExPaSy (www.expasy.org).

Table 2. Estimated CH-PO change for some of the enzymes reported in Table 1, after spontaneous deamidation

Protein	PDB code	Total AA	KRDE	STQN	CH-PO	deamidable Asn	CH-PO (deamid.)
Aldolase	1ADO	248	58	46	4.8	1	5.64
Calbindin	4ICB	79	28	15	16.45	1	18.99
Cytochrome <i>c</i>	2GIW	105	33	18	14.28	2	18.09
Fatty acid-binding protein	1LFO	127	37	28	7.09	1	8.66
Fibroblast growth factor	2AFG	140	36	29	5	1	6.43
Hemoglobin	1A3N	142	26	25	0.70	7	10.56
Hypoxanthine guanine phosphoribosyl transferase	1BZY	217	58	33	11.5	1	12.44
Triose phosphate Isomerase	1HTI	248	62	42	8.06	2	9.67

Table reports data for 8 proteins selected from Table 1: PDB code (www.rcb.org); total number of aminoacids (AA); content in charged (KRDE) and polar (STQN) AA; CH-PO value; number of deamidable Asn reported in Robinson web site (www.deamidation.org); change in CH-PO after deamidation of deamidable Asn.

above, CH-PO of deamidated proteins increases. In fact, a polar AA (Asn or Gln) turns into a charged AA (Asp or Glu). Considering that an elevated CH-PO value renders a protein prone to inactivation by dc exposure (Calzia et al. 2009), and that this electro-inactivation is often accompanied by a change in tertiary structure, it may be supposed that the more highly charged products of deamidation at Asn become sensitive to endogenous currents (Calzia et al. 2009). These currents may produce an “electro-stretch” and unfolding of the deamidated protein (Calzia et al. 2009) which is eventually proteolysed (Fig. 1). A degradation

of the unfolded proteins may explain why Robinson et al. did not find the expected amount of deamidated proteins within the cells (Robinson 2002).

Do deamidated misfolded proteins undergo proteolysis?

An inverse correlation was found between protein Asn + Gln content and their stability (Rogers and Rechsteiner 1988). Deficiency of the repair cytosolic enzyme L-isoaspartate (D-aspartate) O-methyltransferase (PIMT, EC 2.1.1.77),

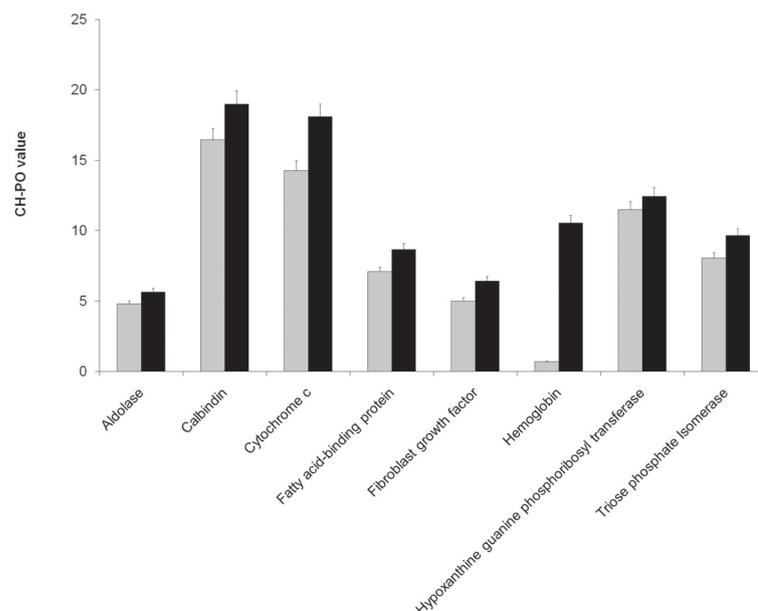


Figure 3. Estimated change in CH-PO of some enzymes after spontaneous deamidation. Figure reports CH-PO value (grey columns) and change in CH-PO after Asn deamidation (black columns) of some proteins used in the paper Robinson (2002).

that converts altered aspartyl residues to normal L-aspartyl residues (Ryttersgaard et al. 2002), results in the accumulation of altered proteins with fatal consequences in knockout mice (Kim et al. 1999). PMIT cannot restore the amide group to Asn residues (Ota and Clarke 1989), but converts the isopeptide to a normal one, that can be hydrolyzed (Galletti et al. 1995). By contrast, an extension of life up to 30% for *Drosophila* overexpressing PIMT was reported (Chavous et al. 2001).

Studies from Dice and Goldberg (1975) on protein turnover *in vivo* showed that acidic proteins are degraded faster. Figure 2 shows that the pI values measured by electrophoresis of some of the proteins in the paper by Dice and Goldberg (1975) appear lower than the theoretic ones, calculated according to ExPaSy Proteomic Server (see Table 1), as though the proteins were already deamidated *in vivo*. The proteins for which the authors measured the most elevated turnover rates were those possessing the lower pI values. Deamidated proteins become more acidic at physiological pH, and their CH-PO rises. Interestingly, CH-PO values of the proteins that displayed elevated turnover rates were over 5.

Figure 3 shows that the CH-PO value of some proteins reported in Robinson (2002) always rises over the mean value for the mesophyle proteins after deamidation of the deamidable Asn. The number of deamidable Asn for each protein was obtained by a query with the PDB identifier in the CD and ID Database (www.deamidation.org). Table 2 shows data that served to build Fig. 2.

May be one cause of cell ageing the removal of some spontaneously deamidated proteins after irreversible loss of ordered structure? Elucidation of these aspects may be an essential step towards an understanding of key biological processes.

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