Deamidation of human proteins

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Deamidation of asparaginyl and glutaminyl residues causes time-dependent changes in charge and conformation of peptides and proteins. Quantitative and experimentally verified predictive calculations of the deamidation rates of 1,371 asparaginyl residues in a representative collection of 126 human proteins have been performed. These rates suggest that deamidation is a biologically relevant phenomenon in a remarkably large percentage of human proteins.

in vivo deamidation | asparaginyl residues

Deamidation of asparaginyl (Asn) and glutaminyl (Gln) residues to produce aspartyl (Asp) and glutamyl (Glu) residues causes structurally and biologically important alterations in peptide and protein structures. At neutral pH, deamidation introduces a negative charge at the reaction site and can also lead to structural isomerization. Early work established that deamidation occurs *in vitro* and *in vivo*, and that the rates of deamidation depend on primary sequence, three-dimensional (3D) structure, pH, temperature, ionic strength, buffer ions, and other solution properties (1–11). It has been hypothesized (3, 5, 7, 12, 13) that Asn and Gln may serve, through deamidation, as molecular clocks which time biological processes such as protein turnover, homeostatic control, and organismic development and aging, as well as mediators of postsynthetic production of new proteins of unique biological value.

Deamidation has been observed and characterized in a wide variety of proteins. It has been shown to regulate some time-dependent biological processes (8, 9) and to correlate with others, such as development and aging. There are many reports of deamidation under physiological conditions in proteins of biological significance. For examples, see refs. 14–18.

Extensive evidence suggests that deamidation of Asn at neutral pH usually proceeds through a cyclic imide reaction mechanism (19–21). Sometimes the Asp produced by deamidation is isomerized to isoAsp. The *in vivo* reversal of this isomerization has been widely reported, but reversal of deamidation itself and of the introduced negative charge has not been observed.

Deamidation rates depend on the amino acid residues near Asn and Gln in the peptide chain with sequence-determined deamidation half-times at neutral pHs and 37°C in the range of 1–500 days for Asn and 100–>5,000 days for Gln (7, 13).

Sequence-determined Asn and Gln deamidation rates are modulated by peptide and protein 3D structures. Deamidation of peptides is observed at both Asn and Gln, largely in accordance with sequence-controlled rates. Deamidation of proteins, which is usually slowed by 3D structure, occurs primarily at Asn except in very long-lived proteins where Gln deamidation is also observed. In a few instances, 3D structure has been reported to increase deamidation rate.

The deamidation rates of individual Asn residues in a protein can be reliably predicted as a result of two recent advances. First, the sequence-controlled Asn deamidation rates of most of the 400 possible near-neighbor combinations in pentapeptide models have been measured (13), and the relevance of this rate library has been established (22). Second, these rates and the 3D structures of proteins with well characterized deamidations have been combined to produce a computation method that correctly predicts the deamidation rates of most Asn residues for which the 3D structure is known (23). This method is more than 95% reliable in predicting relative deamidation rates of Asn residues within a single protein and is also useful for the prediction of absolute deamidation rates.

It is, therefore, now possible to compute the expected deamidation rate of any protein for which the primary and 3D structures are known, except for very long-lived proteins. These proteins require measurement of the 400 Gln pentapeptide rates.

Materials and Methods

Calculation Method. The Brookhaven Protein Data Bank (PDB) was searched to select 126 human proteins of general biochemical interest and of known 3D structure without bias toward any known data about their deamidation, except for 13 proteins (as noted in Table 1) where deamidation has been measured.

The deamidation half-time of each of the 126 proteins was obtained by first computing the deamidation coefficients (C_D) of each Asn and then combining these values into the deamidation index (I_D) by the methods reported (23).

The deamidation coefficient, C_D, is defined as $C_D = (0.01)(t_{1/2})(e^{f(C_m,C_{Sn},S_n)})$, where $t_{1/2}$ is the pentapeptide primary structure half-life (13), C_m is a structure proportionality factor, C_{Sn} is the 3D structure coefficient for the nth structure observation, S_n is that observation, and $f(C_m,C_{Sn},S_n) = C_m[(C_{S_1})(S_1) + (C_{S_2})(S_2) + (C_{S_3})(S_3) - (C_{S_{4,5}})(S_4)/(S_5) + (C_{S_6})(S_6) + (C_{S_7})(S_7) + (C_{S_8})(S_8) + (C_{S_9})(S_9) + (C_{S_{10}})(1 - S_{10}) + (C_{S_{11}})(5 - S_{11}) + (C_{S_{12}})(5 - S_{12})]$. The structure observations, S_n, are those that impede deamidation, including hydrogen bonds, α-helices, β-sheets, and peptide inflexibilities.

For Asn in an α-helical region:

S_1 = distance in residues inside the α-helix from the NH_2 end, where $S_1 = 1$ designates the end residue in the helix, 2 is the second residue, and 3 is the third. If the position is 4 or greater, $S_1 = 0$.

S_2 = distance in residues inside the α-helix from the COOH end, where $S_1 = 1$ designates the end residue in the helix, 2 is the second residue, and 3 is the third. If the position is 4 or greater or $S_1 \neq 0$, then $S_2 = 0$.

$S_3 = 1$ if Asn is designated as completely inside the α-helix because it is 4 or more residues from both ends. If the Asn is completely inside, $S_3 = 1$, $S_1 = 0$, and $S_2 = 0$. If $S_1 \neq 0$ or $S_2 \neq 0$, then $S_3 = 0$.

For flexibility of a loop including Asn between two adjacent antiparallel β-sheets:

S_4 = number of residues in the loop.

S_5 = number of hydrogen bonds in the loop. $S_5 \geq 1$ by definition.

For hydrogen bonds:

S_6 = the number of hydrogen bonds to the Asn side chain C=O group. Acceptable values are 0, 1, and 2.

Abbreviation: 3D, three-dimensional.

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Table 1. Computed deamidation half-times for 126 human proteins in pH 7.4, 37°C, 0.15 M Tris·HCl buffer

Protein	1/2 Life, days	1/10 Life, days	Protein	1/2 Life, days	1/10 Life, days
Uracil-DNA glycosylase (1LAU)	1.0	0.15	Proinsulin (1EFE)	110	17
Uroporphyrinogen decarboxylase (1URO)	1.0	0.15	Mitogen-activated protein kinase P38 (1WFC)	110	17
Transaldolase (1F05)	1.4	0.21	Glutathione reductase (1BWC)	120	18
Urokinase-type plasminogen activator (1LMW)	1.7	0.26	Ribonuclease 4 (1RNF)	130	20
Purine nucleoside phosphorylase (1ULA)	1.8	0.27	Aldose reductase (1EL3)	130	20
Growth hormone receptor (1A22)	2.4	0.36	α-Lactalbumin (1B9O)	130	20
Peptidyl-prolyl cis-trans isomerase (1F8A)	2.4	0.36	Ornithine transcarbamoylase (1OTH)	130	20
Thymidylate synthase (1HW3)	2.7	0.41	Malic enzyme (1EFK)	140	21
Procathepsin B (3PBH)	2.9	0.44	Glucose-6-phosphate 1-dehydrogenase (1QKI)	140	21
D-Glyceraldehyde-3-phosphate dehydrogenase (3GPD)	4.2	0.64	Procarboxypeptidase A2 (1AYE)	150	23
Karyopherin β2 (1QBK)	5.3	0.81	Apoptosis regulator bax (1F16)	170	26
Glutathione *S*-transferase (12GS)	5.3	0.81	Ornithine decarboxylase (1D7K)	170	26
N-acetylgalactosamine-4-sulfatase (1FSU)	6.1	0.93	UDP-galactose 4-epimerase (1EK6)	180	27
Fructose bisphosphate aldolase (4ALD)	7.6	1.2	Stem cell factor (1EXZ)*	180	27
Intestinal fatty acid binding protein (3IFB)	7.6	1.2	Hypoxanthine guanine phosphoribosyltransferase (1BZY)*	180	27
Cyclophilin A (1AWQ)	8.7	1.3	Electron transfer flavoprotein (1EFV)	190	29
Vascular endothelial growth factor (2VPF)*	10	1.5	Phenylalanine hydroxylase (1DMW)	220	33
Inositol monophosphatase (1IMB)	15	2.3	Annexin V (1ANX)	220	33
Pancreatic inhibitor variant 3 (1CGI)	16	2.4	Platelet factor 4-HPF4 (1RHP)	230	35
D-Glucose 6-phosphotransferase (1HKC)	16	2.4	Insulin (2HIU)*	260	40
Myeloperoxidase (1MHL)	16	2.4	Prethrombin2 (1HAG)	260	40
α-Chymotrypsinogen (1CGI)	16	2.4	Interleukin-4 (2CYK)	270	41
Lysophospholipase (1LCL)	16	2.4	Interleukin-1β (2I1B)	280	43
Interleukin-16 (1I16)	17	2.6	Neutrophil (gelatinase) (1DFV)	290	44
C-AMP-dependent kinase A (1CMK)	19	2.9	*O*6-alkylguanine-DNA alkyltransferase (1EH6)	300	46
Pepsinogen (1HTR)	20	3.0	Glucosamine-6-phosphate deaminase isomerase (1D9T)	320	49
Angiogenin (1A4Y)*	21	3.2	Quinone reductase type 2 (1QR2)	330	50
Fibroblast growth factor (2AFG)*	21	3.2	Nad(P)H dehydrogenase (1QBG)	350	53
Gastric lipase (1HLG)	21	3.2	Serum albumin (1E7G)	360	55
Calmodulin (1CTR)	21	3.2	Plasminogen activator inhibitor-1 (1C5G)	370	56
Bone morphogenetic protein 7 (1BMP)	21	3.2	T cell surface glycoprotein CD4 (1CDJ)*	380	58
Acetylcholinesterase (1F8U)	23	3.5	α-Thrombin (1A3E)	380	58
Retinol binding protein (1BRQ)*	24	3.6	Eosinophil cationic protein (1QMT)	430	65
Catalase (1QQW)	25	3.8	Ribonuclease inhibitor (1A4Y)	450	68
Dihydrofolate reductase (1DRF)	25	3.8	Transforming growth factor-β two (1KLA)	460	70
Interleukin-10 (2ILK)	25	3.8	Thioltransferase (1JHB)*	470	71
Farnesyltransferase (1EZF)	26	4.0	Profilin 1 (1FIL)	480	73
S-adenosylhomocysteine hydrolase (1A7A)	28	4.3	Lithostathine (1LIT)	490	74
Procathepsin K (1BY8)	28	4.3	Phosphatidylethanolamine binding protein (1BD9)	680	100
3-Methyladenine DNA glycosylase (1BNK)	35	5.3	Dihydroorotate dehydrogenase (1D3G)	720	110
Medium chain acyl-coa dehydrogenase (1EGE)	36	5.5	Quinone reductase (1D4A)	750	110
Homeobox protein PAX-6 (6PAX)	39	5.9	Hemoglobin (1A3N)*	780	120
α1-Antitrypsin (1QLP)	40	6.1	Retinoic acid receptor (1BY4)	860	130
Carbonic anhydrase I (1HCB)	45	6.8	Psoriasin (1PSR)	890	140
GTP-binding protein (1DOA)	45	6.8	ADP-ribosylation factor 6 (1E0S)	>1000	150
Ferritin (2FHA)	46	7.0	Lectin L-14-II (1HLC)	>1000	180
Procathepsin L (1CS8)	48	7.3	Nucleoside diphosphate kinase (1NUE)	>1000	210
Growth hormone (1HGU)*	51	7.8	L-3-Hydroxyacyl-CoA dehydrogenase (1F0Y)	>1000	230
Triose phosphate isomerase (1HTI)*	52	7.9	Interleukin 2 (3INK)*	>1000	230
Interleukin-6 (1IL6)	56	8.5	Transthyretin (1DVQ)	>1000	290
DNA polymerase β (1BPX)	58	8.8	Single-stranded DNA binding protein (3ULL)	>1000	290
Glutathione synthetase (2HGS)	58	8.8	Protein kinase C interacting protein 1 (1KPA)	>1000	300
Fructose-1,6-bisphosphatase (1FTA)	59	9.0	GTPase ran (1QBK)	>1000	380
CDK2 kinase (1BUH)	65	9.9	Annexin III (1AXN)	>1000	430
Ribonuclease A (1AFK)	66	10	Fk506-binding protein (1D6O)	>1000	710
Ap endonuclease (1BIX)	72	11	Interleukin-5 (1HUL)	>1000	760
Carbonic anhydrase IV (1ZNC)	72	11	Heme oxygenase (1QQ8)	>1000	780
Branched-chain α-keto acid dehydrogenase (1DTW)	81	12	Histone H2A.Z (1F66)	>1000	>1000
Argininosuccinate lyase (1AOS)	83	13	Copper transport protein ATOX1 (1FEE)	>1000	>1000
Creatine kinase (1QK1)	84	13	17 β-Hydroxysteroid dehydrogenase (1DHT)	>1000	>1000
Carbonic anhydrase II (1BV3)	90	14	Myoglobin (2MM1)	>1000	>1000
Interleukin-8 (1IL8)	95	14	Ubiquitin (1D3Z)	>1000	>1000
Dihydropteridine reductase (1HDR)	100	15	Granulocyte colony-stimulating factor (1RHG)	>1000	>1000

The proteins were selected without regard to reported deamidation except for 13 proteins that were among those used to develop and test the calculation method (23, 26–28) and are designated with an asterisk in the table.

S_7 = the number of hydrogen bonds to the Asn side chain NH_2 group. Acceptable values are 0, 1, and 2.
S_8 = the number of hydrogen bonds to the backbone N in the peptide bond on the COOH side of Asn. Hydrogen bonds counted in S_6 or S_7 are not included. Acceptable values are 0 and 1. This nitrogen is used in the five-membered succinimide ring.
S_9 = additional hydrogen bonds, not included in S_6, S_7, and S_8, that would need to be broken to form the succinimide ring.

For Asn situated so that no α-helix, β-sheet, or disulfide bridge structure is between the Asn and the end of the peptide chain:

S_{10} = 1 if the number of residues between the Asn and the nearest such structure is 3 or more. If the number of intervening residues is 2, 1, or 0, or if the Asn is not between structure and chain end, then $S_{10} = 0$.

If the Asn lies near to any α-helix, β-sheet, or disulfide bridge structures:

S_{11} = the number of residues between the Asn and the structure on the NH_2 side, up to a maximum of 5. Values of 0, 1, 2, 3, 4, and 5 are acceptable.
S_{12} = the number of residues between the Asn and the structure on the COOH side, up to a maximum of 5. Values of 0, 1, 2, 3, 4, and 5 are acceptable.

Hydrogen bonds are accepted if the bond length is 3.3 Å or less and there is room in the structure to accommodate the van der Waals radius of the hydrogen. All primary structure $t_{1/2}$ values are those published (13), except for Asn with carboxyl-side Pro, Asn, or Gln, and Asn without a free amide as a result of binding to metals or other moieties. Estimated values of $t_{1/2}$ of 500, 40, 60, and 500 days are used for AsnPro, AsnAsn, AsnGln, and bound Asn, respectively.

The coefficients C_m and C_{Sn} were optimized by means of the D_P method (23–25) with an increased set of proteins (26–29). D_P is a measure of the percentage accuracy in classifying the relative deamidation rates of Asn residues in a set of proteins (23). Proteins added to the original set (23) and their Protein Data Bank numbers were bovine DNase I (2DNJ), human hirudin (4HTC), bovine calmodulin (1A29), and human vascular endothelial growth factor (2VPF). Human T cell surface glycoprotein CD4 (1CDJ) was omitted. The Asn 3D environments in all 31 of the calibration proteins were examined and retabulated. These 31 proteins include all of the proteins suitable for this purpose that we have found in the research literature.

The optimized values were $C_m = 0.48$, $C_{S1} = 1.0$, $C_{S2} = 2.5$, $C_{S3} = 10.0$, $C_{S4,5} = 0.5$, $C_{S6} = 1.0$, $C_{S7} = 1.0$, $C_{S8} = 3.0$, $C_{S9} = 2.0$, $C_{S10} = 2.0$, $C_{S11} = 0.2$, and $C_{S12} = 0.7$. These values are identical to those found in ref. 23. The deamidation resolving power (D_P) was found to be 95.4%.

The protein deamidation index is defined as $I_D = [\Sigma (C_{Dn})^{-1}]^{-1}$, where C_{Dn} is C_D for the nth Asn residue. Therefore, $(100)(I_D)$ is an estimate of the initial single-residue deamidation half-time for the protein with all Asn residues considered.

Comparison of Calculated Rates with Experimental Rates

The Medline and Citation Index databases were searched for all proteins in which the deamidation rates of identified Asn residues have been reported for 37°C solutions with pH at or near 7.4. Reports were found for a total of 10 individual Asn and 3 combinations of Asn residues in 10 different protein types (17, 18, 23, 29–35). These include 7 proteins that are in the human set of 126 and 3 proteins from other species.

The names, Protein Data Bank numbers, Asn residue position, and computed $(100)(I_D)$ values, respectively, for these proteins are rabbit aldolase: 1ADO, 360, 8.3; human vascular endothelial growth factor: 2VPF, 10, 10; *Escherichia coli* Hpr-phosphocarrier protein: 1HDN, 12–38, 89–22; human fibroblast growth factor: 2AFG, 7, 64; human angiogenin: 1B1I, All, 28; human retinal binding protein: 1BRQ, All, 29; human growth hormone (GH): 1HGU, All, 51; human triose phosphate isomerase: 1HTI, 71, 78; bovine ribonuclease A: 1AFK, 67, 84; and human hemoglobin: 1A3N, with Asn mutants at α50-β80-β82, 47-190-184.

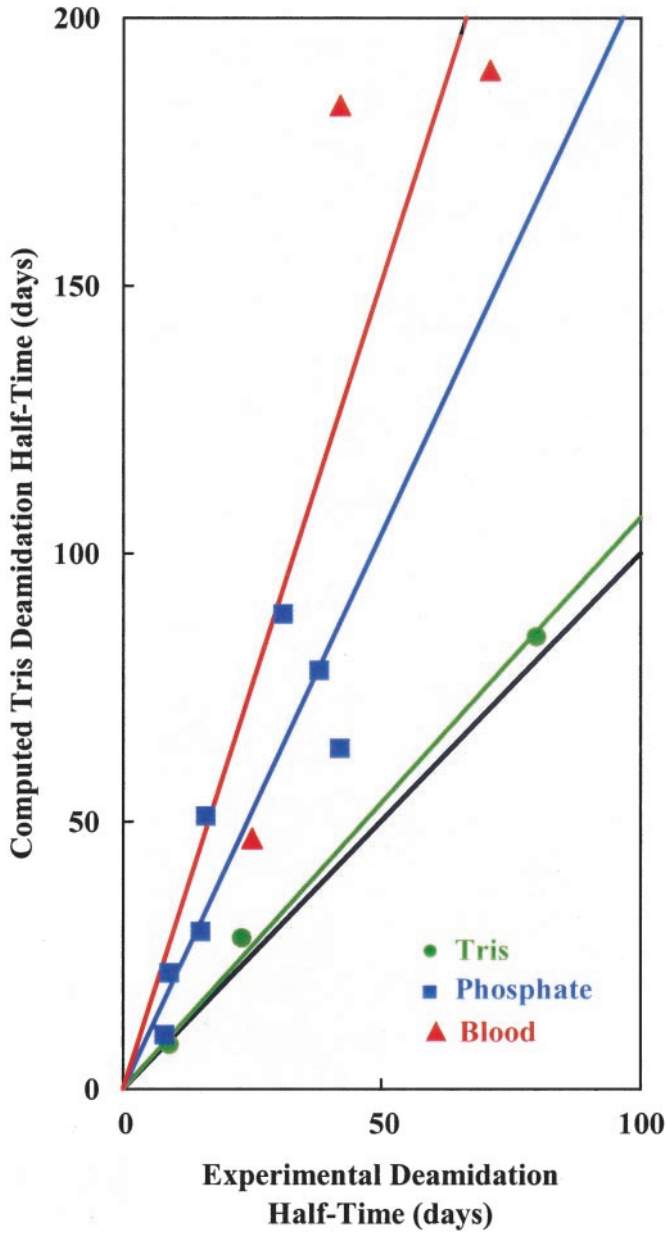

Fig. 1. Calculated single deamidation half-times for 10 individual Asn and 3 combinations of Asn residues in 10 different protein types vs. the corresponding experimentally observed deamidation half-times (17, 18, 23, 29–35). Experiments were *in vitro* in Tris and phosphate buffers and *in vivo* in human blood. Buffer conditions in Tris and phosphate varied among these investigations but were comparable to pH 7.4, 37°C, 0.15 M. Some of the scatter in the figure is probably the result of these variations. If the calculated values and experimental values were identical, the points would lie on the solid black line, as do the values determined in Tris buffers. Catalysis of deamidation is higher by phosphate than by Tris and may be even higher in erythrocytes.

Fig. 1 compares the computed half-times for pH 7.4, 37°C, 0.15 M Tris·HCl buffer with the experimentally observed (17, 18, 29–35) values. The computed values compare favorably with the

experimental values in Tris buffer. In phosphate buffer, the experimental deamidation rates are, on average, 2-fold higher than calculated, and the 3 *in vivo* human blood values average 3-fold higher. This result is entirely as expected because deamidation at neutral pH is subject to catalysis by solution ions. Tris is a very mild catalyst of deamidation. Phosphate is a much stronger catalyst of deamidation in peptides (6, 11) and proteins (17) as compared with Tris. Tissue culture medium contains components even more catalytic of deamidation than phosphate (36). Least-squares lines as shown in Fig. 1 give experimental deamidation rates relative to the computed values in Tris, phosphate, and *in vivo* blood erythrocytes of 1.06, 2.07, and 3.01, respectively.

The agreement between the calculated values and Tris experimental values in Fig. 1 does not arise from computational forcing. The computational method (23) uses experimental sequence-determined pentapeptide deamidation rates in Tris buffer and a parametric 3D structure function with adjustable constants. The optimization method (23–25) for these constants used only the ordered Asn residue instabilities in a wide variety of proteins and buffer types. No experimental absolute deamidation rates were used. The agreement arises because the computational method correctly estimates the relative primary and 3D contributions to the deamidation rate of each Asn, and the primary rates were experimentally determined in Tris.

Results and Discussion

Averaged over all 1,371 Asn, the contributions to the deamidation reaction activation energy from primary and 3D structures are about equal, although they vary widely for individual Asn residues. The average relative deamidation rates of Asn within single proteins in this 126-protein set are 60% determined by primary and 40% by 3D structure, which are the same proportions found for a different 24-protein set (23). The cumulative distribution function of the calculated first-order rate constants for deamidation of the 1,371 Asn residues is shown in Fig. 2*a*.

The computed single deamidation half-times in pH 7.4, 37°C, 0.15 M Tris·HCl buffer for the 126 human proteins are shown in Table 1. Table 2 summarizes, on the basis of Table 1, the extent to which deamidation is expected to occur within this set of 126 proteins.

The percentages of deamidation in living tissues are probably higher than shown in Tables 1 and 2. Physiological fluids contain many inorganic, organic, and biochemical substances with deamidase activity. We know of no reported instance, *in vivo* or *in vitro*, of an experimentally measured protein deamidation rate that is slower than its computed Tris rate. All reported rates are the same or faster. There are two instances of individual proteins (13, 23) in which negative results for the detection of deamidation in specific amides indicates that the rates, if measured, might be slower than calculated.

This deamidation is not a random consequence of the presence of Asn residues in proteins. The fast deamidations summarized in Table 2 result from a set of Asn residues with unusual primary and 3D structures, which comprise about 5% of the total. As illustrated in Fig. 2, most individual Asn deamidation rates are slower. Because a large number of similarly sized partially independent factors determine Asn deamidation rates in proteins, the distribution functions in Fig. 2 would be expected to be Gaussian. Fig. 2*b* shows the deviation from Gaussian caused by the unusual Asn residues.

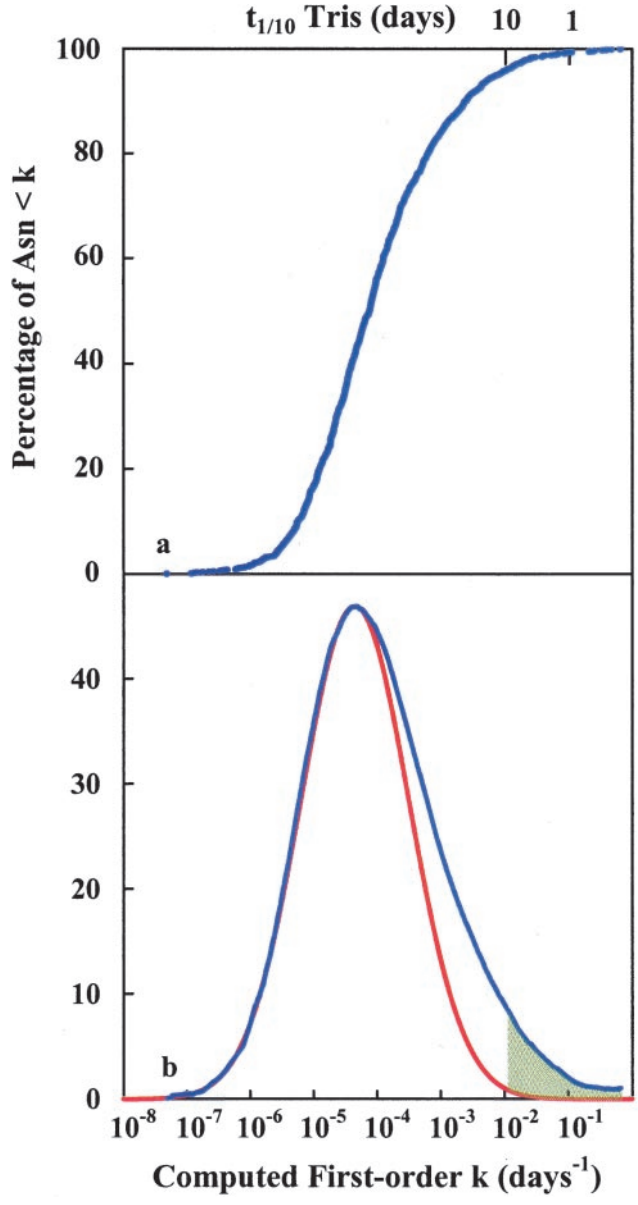

Fig. 2. (*a*) Cumulative distribution function of the calculated first-order rate constants for deamidation of 1,371 Asn residues in 126 human proteins. As indicated, the Asn residues involved in the initial deamidation of these proteins comprise a relatively small part of the complete set. Computed percentages of the Asn residues that are 1/10 deamidated at 1 and 10 days in Tris are 1% and 4%, respectively, as shown. If this deamidation were not of positive biological value, more slowly deamidating sequences and 3D structures could easily have been used. (*b*) Differentiated values of the distribution function in *a* showing the special class of unstable Asn residues present in human proteins. Also shown is a Gaussian function that fits the distribution function except for that part arising from the especially unstable Asn residues. The shaded area contains those Asn residues computed to be one-tenth or more deamidated in 10 days in pH 7.4, 37°C, 0.15 M Tris·HCl. This shaded area for phosphate, physiological fluids, or longer time intervals would be a larger part of the illustrated deviation from Gaussian.

Conclusions

The unstable Asn residues that give rise to the deamidation rates shown in Tables 1 and 2 and Figs. 1 and 2 are apparently preferred over the many stable Asn residues that could easily be genetically specified. As shown in Fig. 2, most Asn residues are far more stable. Moreover, even if it were not a result of preference, this introduction of negative charges into protein structures would do unacceptable biochemical damage unless it was being used for compensating biological purposes.

Although postsynthetic deamidated proteins are often observed in tissue extracts, their production can be obscured. For example, the *in vivo* steady-state concentrations of the deamidated forms of cytochrome *c* are much lower than expected

Table 2. Percentages of human proteins in Table 1 computed to be more than one-tenth or one-half singly deamidated in Tris or phosphate buffer after 1, 5, 10, and 50 days

Days at 37°C pH 7.4	Proteins singly deamidated by >1/10		Proteins singly deamidated by >1/2	
	Tris	Phosphate	Tris	Phosphate
1	10%	13%	1.6%	4%
5	31%	43%	8%	13%
10	43%	56%	13%	20%
50	71%	82%	37%	49%

A phosphate buffer correction of (2)(computed Tris rate) was applied to obtain the phosphate rate of each protein. Percentages produced in physiological solutions may be even higher. Steady-state physiological percentages are lowered by protein turnover.

because they are preferentially degraded (7, 8). Those deamidated forms that are not degraded and, therefore, accumulate in living tissues may have other unique biological purposes. Otherwise, their accumulation would be disadvantageous.

Moreover, the deamidation rates in living tissues are changeable. Through the production of enzymatic deamidases or the control of other physiological parameters that affect the reaction activation energy of deamidation, a living cell could easily increase the overall deamidation rates of its proteins to adapt to changes in physiological circumstances. Decrease of deamidation rates to values below those in Tris summarized in Table 2, however, would be difficult except in specialized structures. Deamidation has been observed in the proteins of many other organisms, too; thus, similar findings may be expected.

In summary, reliable and experimentally verified predictive calculations of the deamidation rates of 1,371 Asn residues in a representative collection of 126 human proteins have been carried out. The results of these calculations show that deamidation of human proteins under physiological conditions is so extensive that it is probably of pervasive and fundamental biological importance. Otherwise, the genetic code would specify stable Asn configurations. Likely uses of deamidation include the timing of biological processes and the postsynthetic production of uniquely useful proteins.

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