Protein Safety: Toxicological Testing of Proteins

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ILSI-IFBiC Task Force 10

In 2008, the International Life Sciences Institute Food Biotechnology Committee (ILSI-IFBiC) formed Task Force 10.

Goal: to develop international consensus recommendations regarding the role of animal toxicology studies in the food safety assessment of biotech crops.

Task Force 10 has met periodically and prepared documents for publication:

- **Toxicological Evaluation of Proteins Introduced into Food Crops (this talk)**
- **Assessing the Utility of Whole Food Studies for the Safety Assessment of Genetically Engineered Crops**

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ILSI-IFBiC Task Force 10

Sue Barlow – UK Consultant

Andrew Bartholomaeus – Food Standards Australia New Zealand

Genevieve Bondy – Health Canada

Amechi Chukwudebe – BASF

Bryan Delaney – Pioneer, a Dupont Company

Bruce Hammond – Retired from Monsanto Company

Corinne Herouet-Guicheney – Bayer SAS

Joseph Jez – Washington University

Daland Juberg – Dow AgroSciences

Hideaki Karaki – Retired from University of Tokyo

John Kough – US EPA

Sue MacIntosh – MacIntosh & Ass.

Wayne Parrott – University of Georgia

Alaina Sauve – Syngenta Biotechnology, Inc.

Kate Walker – ILSI IFBiC

Flavio Zambrone – Planitox, ILSI Brazil

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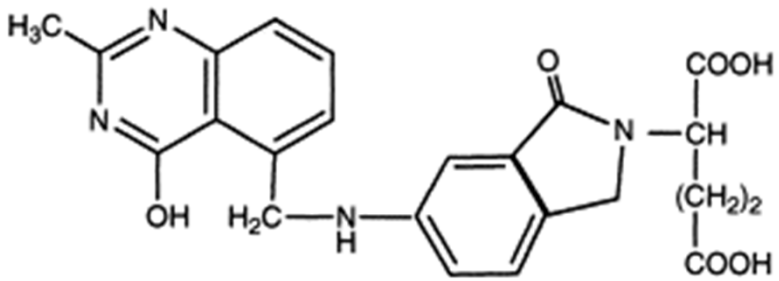
What is being tested?

- **Toxicological evaluations were developed for chemicals, not for proteins (and other macromolecules)**

example: a folate-analog developed as a thymidylate synthase inhibitor (1843U89)



1843U89

Molecular Formula: $C_{27}H_{24}N_4O_6$ Molecular Weight: 500.5 Da (g/mol)

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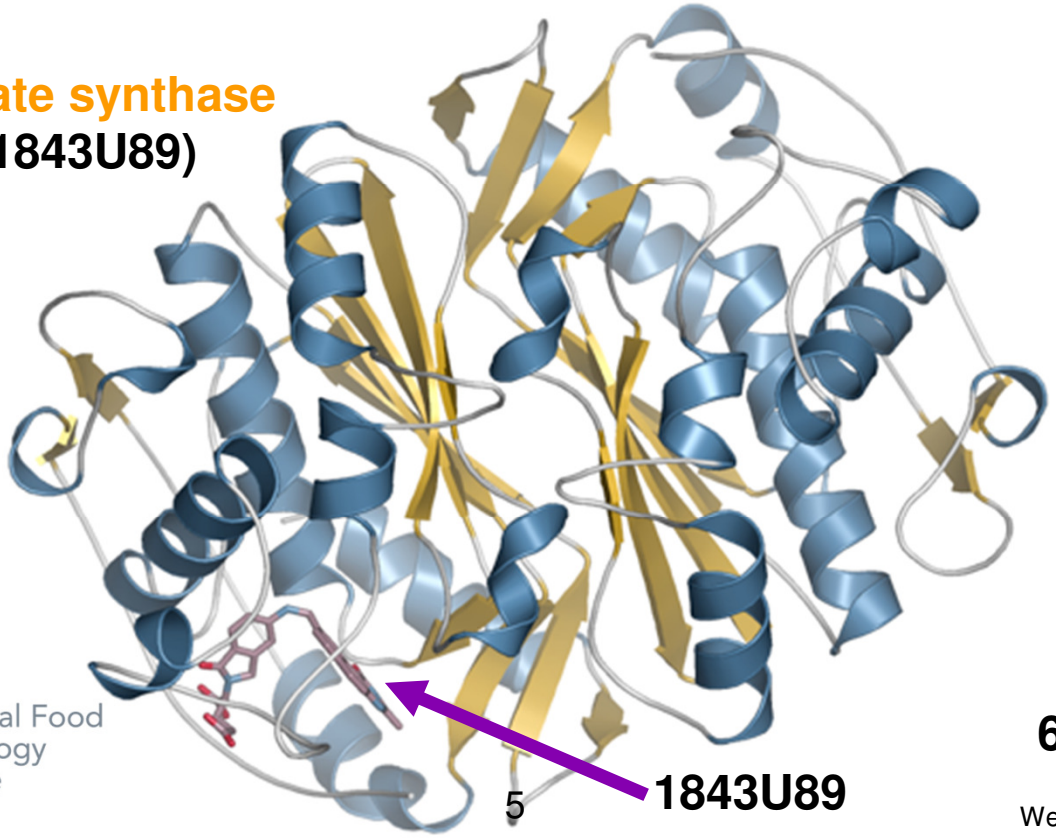
Duch et al (1993) Cancer Res 53, 810

What is being tested?

- **Toxicological evaluations were developed for chemicals, not for proteins (and other macromolecules)**
- ***A protein is not equivalent to a small chemical molecule***
- ***Proteins are nutritional - amino acid source***

bacterial thymidylate synthase
with inhibitor (1843U89)



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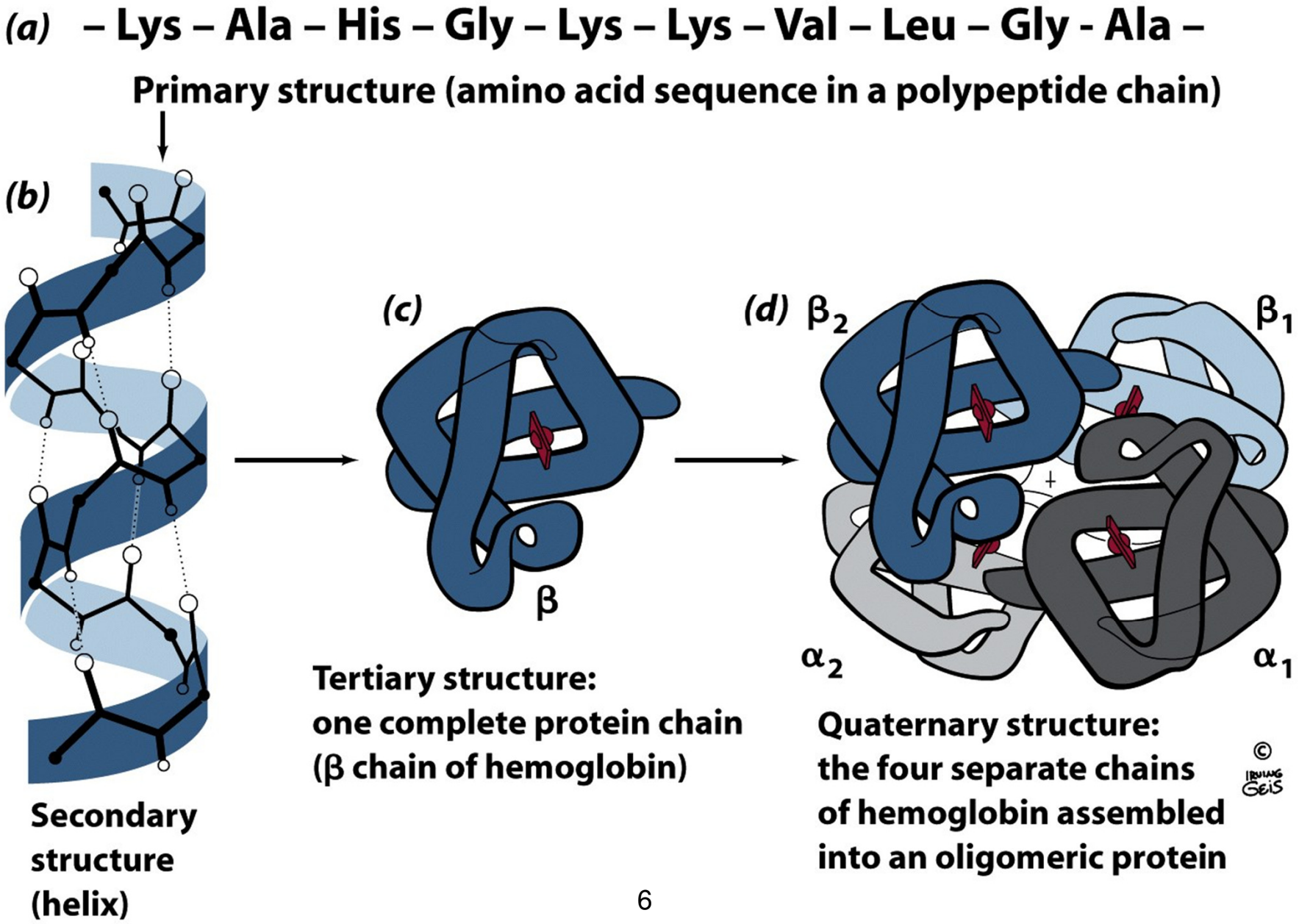
protein is
60,959.2 Da (g/mol)

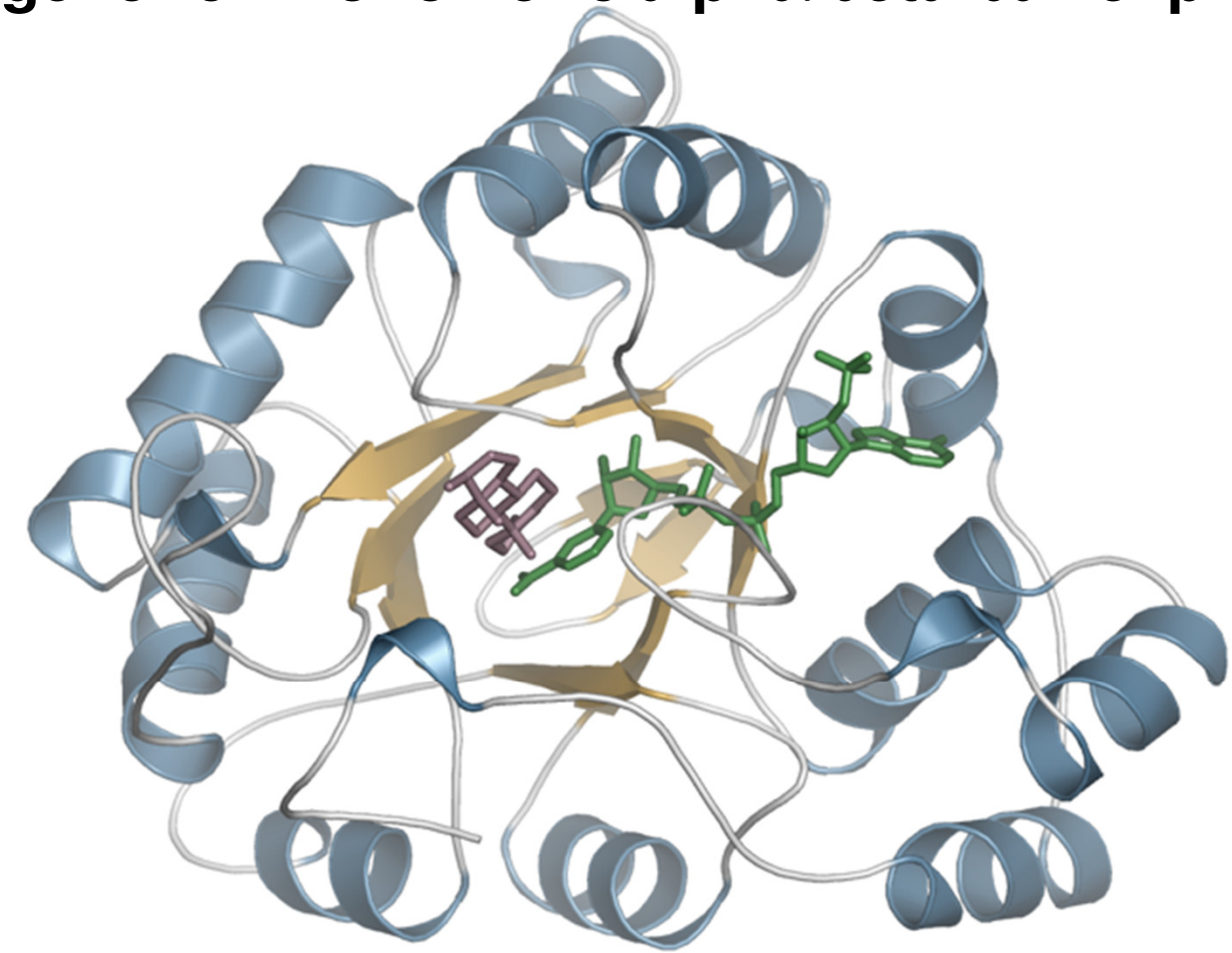
Weichsel et al (1995) PNAS 92, 3493

Proteins 101: Protein Structure



Proteins 101: Protein Structure/Function

a "generic" monomeric alpha/beta barrel protein



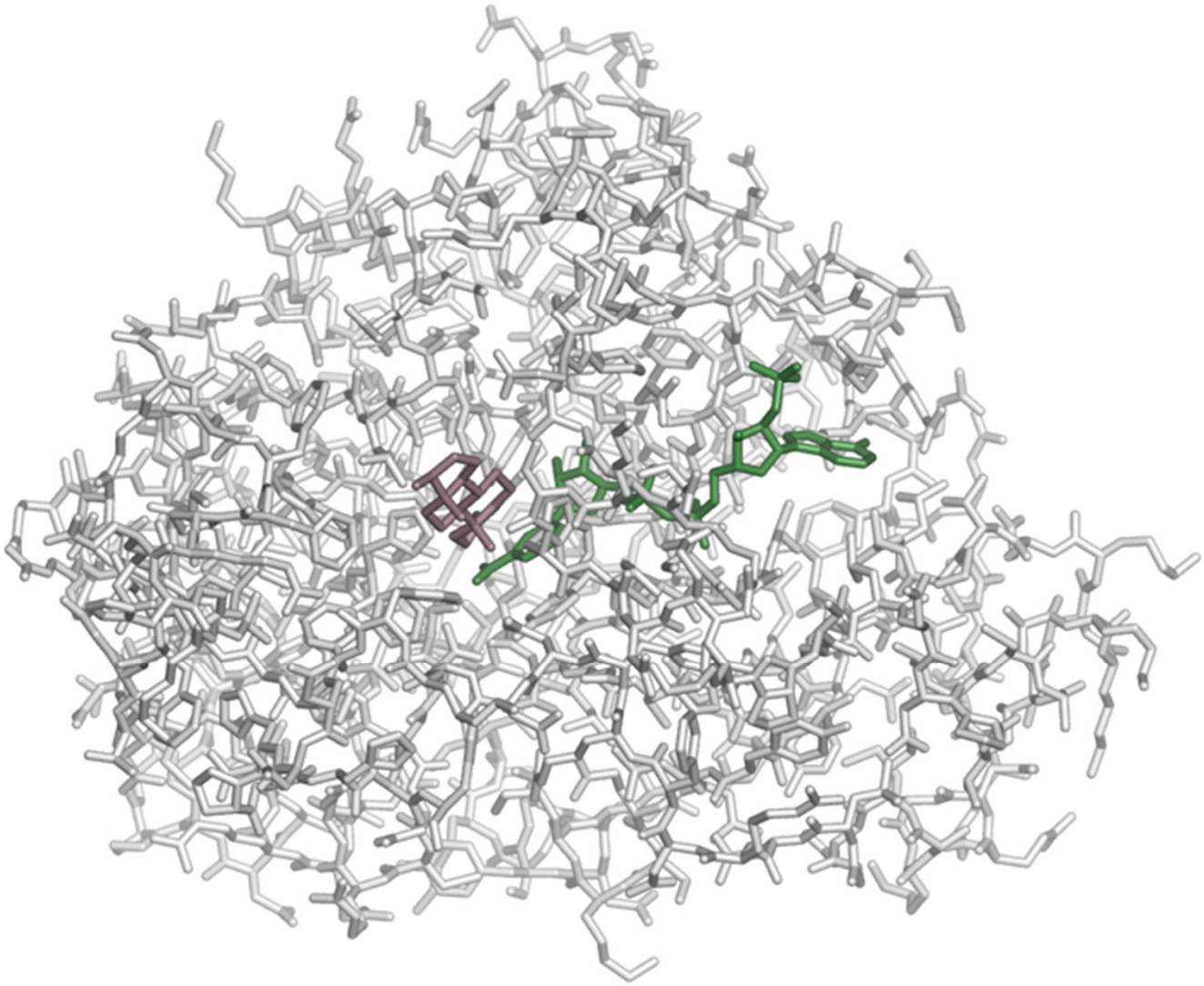
steroid

NAD+

Ribbon Diagram

Bennett et al (1997) Structure 5, 799

Proteins 101: Protein Structure/Function



319 amino acids (2582 C, N, O, S atoms)

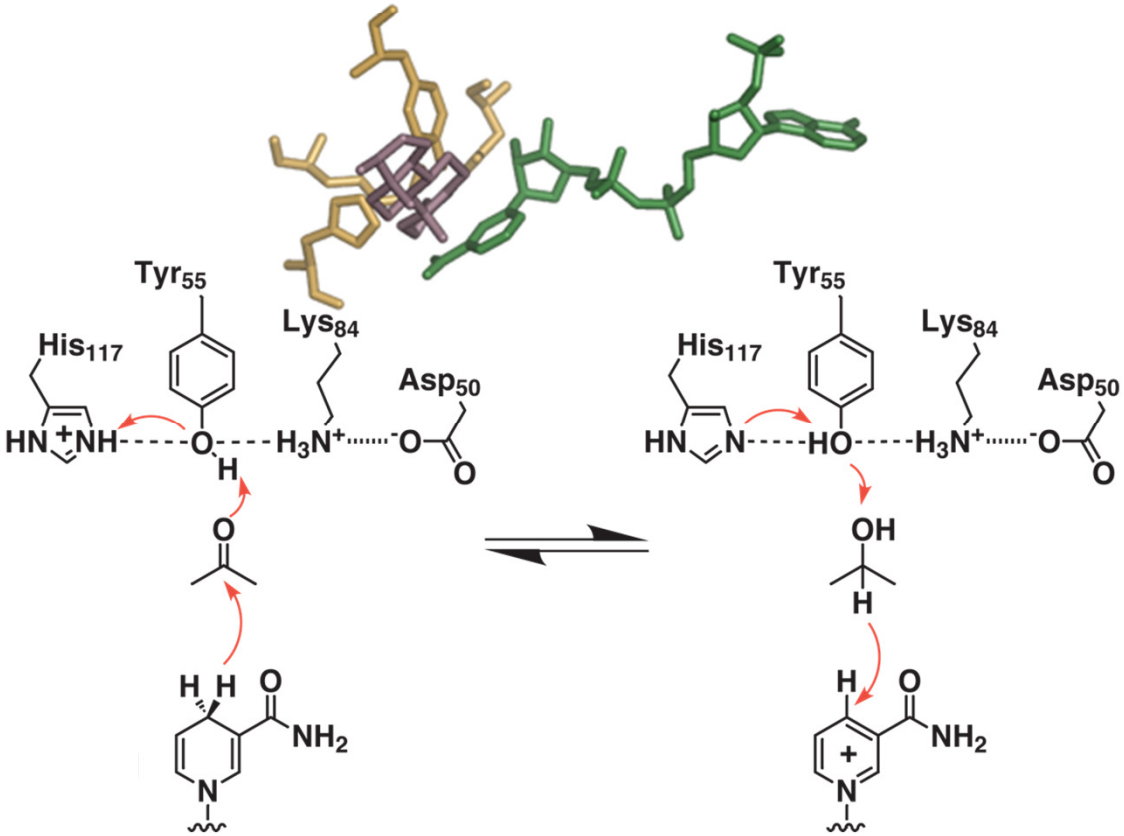
steroid

NAD+

Bennett et al (1997) Structure 5, 799

Proteins 101: Protein Structure/Function

4 residues are required for catalysis



Bennett et al (1997) Structure 5, 799

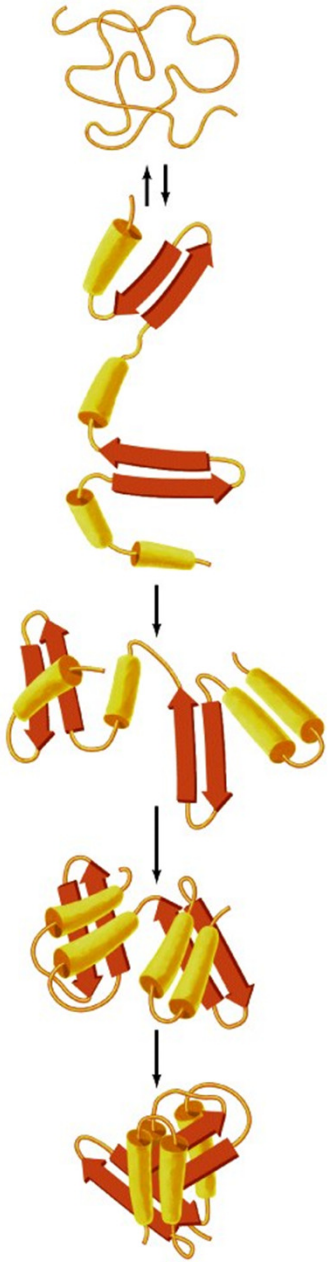
Proteins 101: Protein Folding

unfolded

formation of 2° structures

molten globule

packed & folded



$$\Delta G = \Delta H - T\Delta S$$

free energy *enthalpy* *entropy*

What holds a protein together?

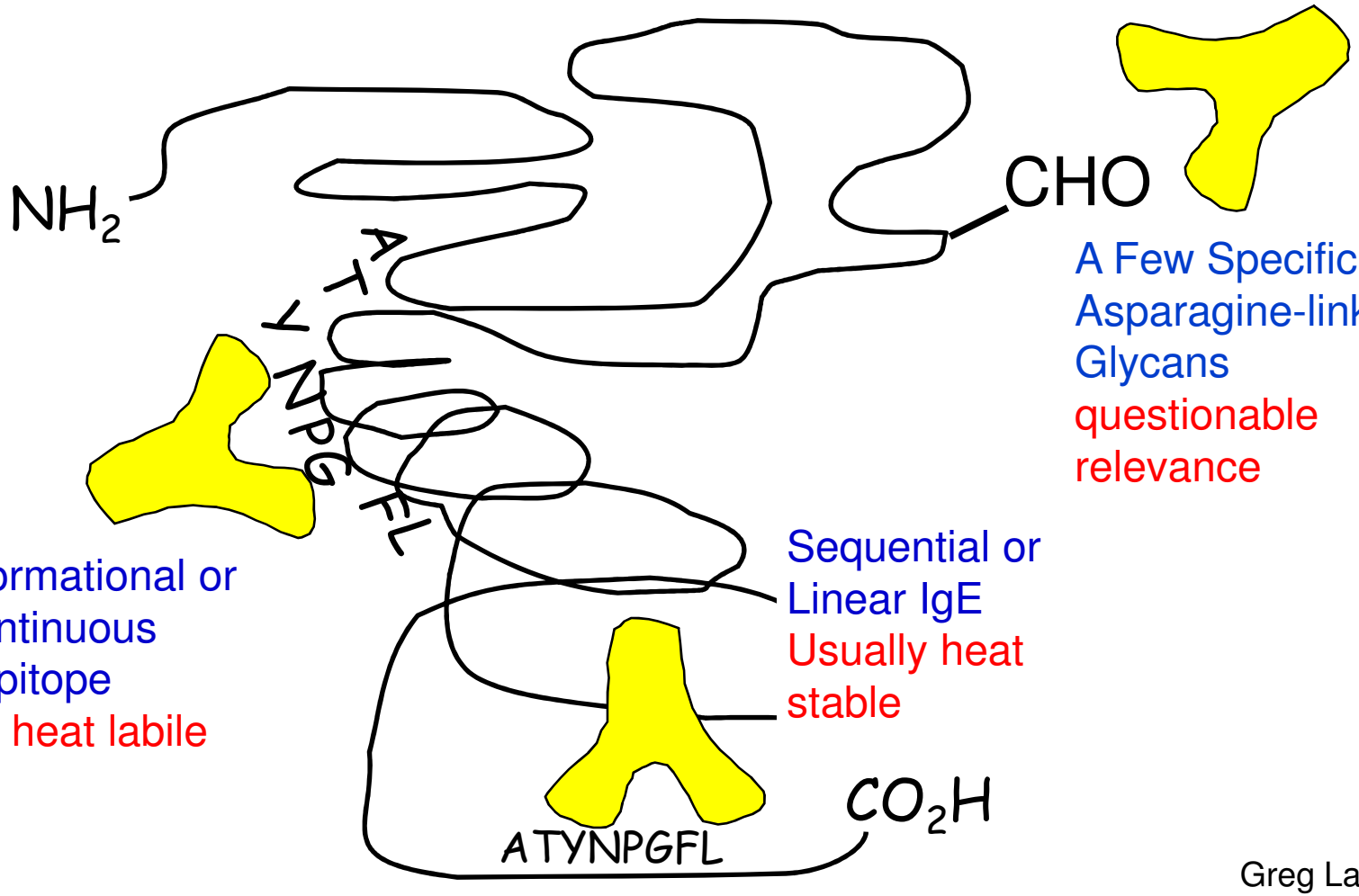
van der Waals interactions
hydrogen bonds
charge-charge interactions
hydrophobic effect

Digestion and Food Processing

proteases/acid hydrolysis
temperature changes
pressure changes
physical sheering
pH changes
drying/solvent changes

Proteins 101: Protein Folding

Example of why proper protein folding matters
1) allergens and epitopes



A Few Specific Asparagine-linked Glycans questionable relevance

Conformational or discontinuous IgE epitope
Often heat labile

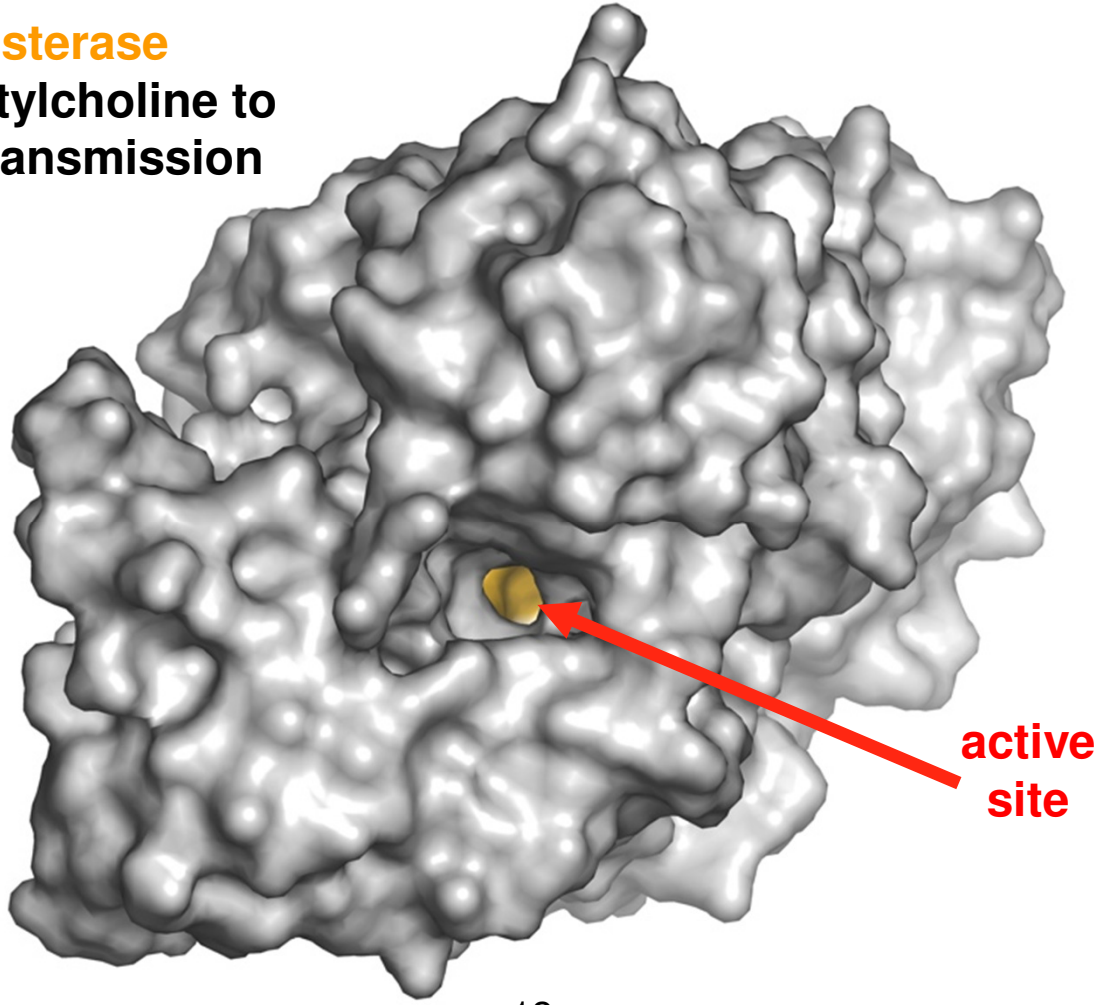
Sequential or Linear IgE
Usually heat stable

Greg Ladics
Dupont

Proteins 101: Protein Folding

Example of why proper protein folding matters
2) protein toxins require structure for mode of action

acetylcholinesterase
breakdown of acetylcholine to terminate neurotransmission



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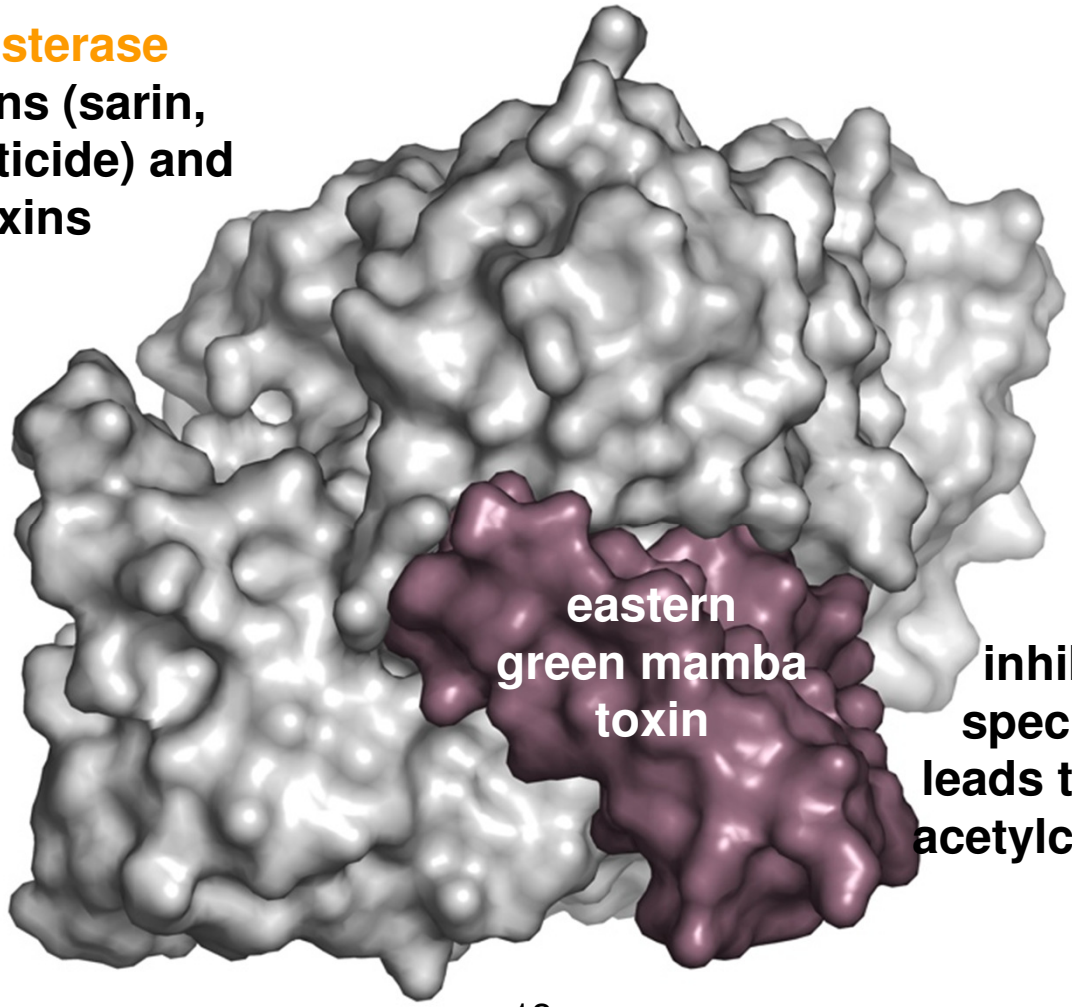
Harel et al (1993) PNAS 90, 9031

Proteins 101: Protein Folding

Example of why proper protein folding matters
2) protein toxins require structure for mode of action

acetylcholinesterase
target of poisons (sarin, malathion insecticide) and protein toxins



inhibition by highly specific protein toxin leads to accumulation of acetylcholine and muscle paralysis

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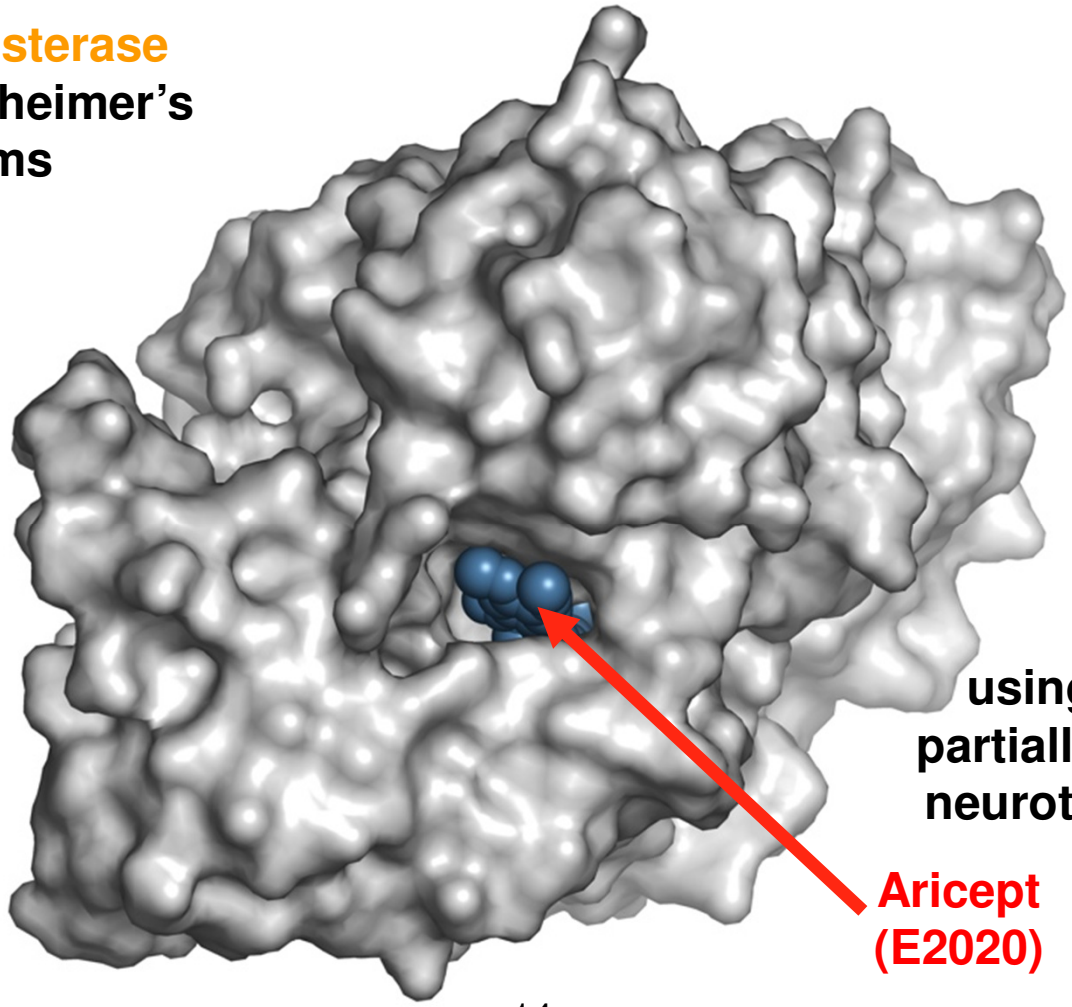
Kryger et al (2000) Acta Cryst D 56, 1385

Proteins 101: Protein Folding

Example of why proper protein folding matters
2) protein toxins require structure for mode of action

acetylcholinesterase
reversal of Alzheimer's symptoms



using a chemical to partially block and raise neurotransmitter levels

Aricept (E2020)

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Kryger et al (1999) Structure 7, 297

CODEX Alimentarius Guidelines on Safety of an Introduced Protein

The assessment of potential toxicity should focus on:

- **amino acid sequence similarity between the protein and known protein toxins and anti-nutrients (bioinformatics)**
- **stability to heat and processing and to degradation in appropriate gastric and intestinal model systems**
- **oral toxicity studies that may need to be carried out if the protein present in food is not similar to proteins that have previously been consumed safely in food**

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CODEX, 2009. Foods derived from modern biotechnology. WHO/FAO.

ILSI TF6 Conclusions on Safety Assessment of Introduced Proteins (2008)

Tier I. Basic Hazard Assessment

- history of safe use (HOSU)
- bioinformatics
- expression level and dietary intake
- mode of action
- *in vitro* digestiblity and heat lability

Tier II. Supplementary Hazard Assessment

- triggered when concerns raised in Tier I
- toxicology studies

proteins not structurally or functionally related to known toxins based on bioinformatics and confirmed to be digestible are less likely to pose a hazard when consumed

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Delaney et al., 2008. Food Chem. Tox. 46, S71-S97

New Issues Considered by TF10 Regarding Protein Safety

- **What is the impact of food processing on potential dietary exposure to functionally active introduced proteins?**
- **For purposes of risk assessment, can the Threshold of Toxicological Concern (TTC) model be applied to proteins?**
- **What are the criteria for assessing "History of Safe Use (HOSU)"?**

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TF10 Protein Safety - New Issues

What is the impact of food processing on potential dietary exposure to functionally active introduced proteins?

- **Protein function depends on maintenance of tertiary structure**
- **Many food crops are processed, which disrupts protein tertiary structure (denaturation)**
- **In vitro heat stability tests indicate that proteins exposed to processing temperatures lose function**
- **Processed food analyses also confirm loss of introduced protein function**

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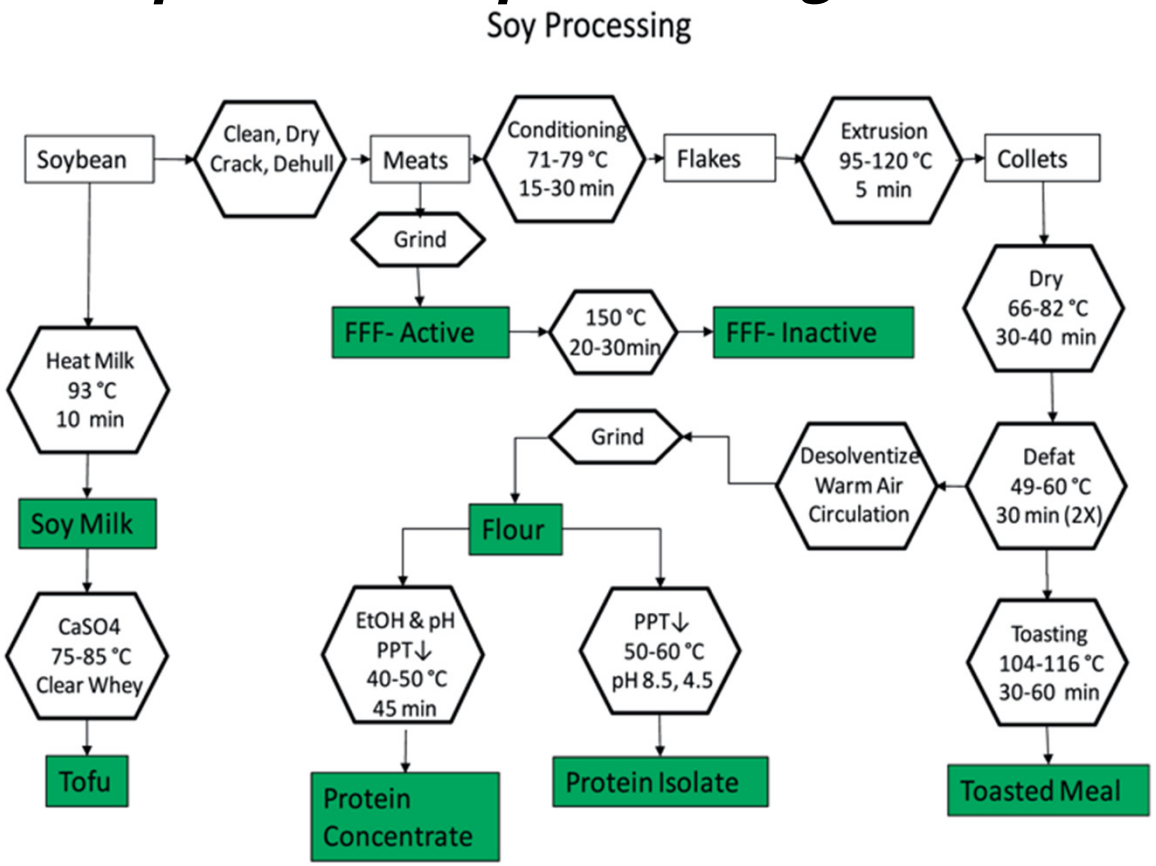
TF10 Protein Safety - New Issues

Example of food processing conditions



processing conditions are not ideal for maintaining structure/function

- **solvents, drying, solution conditions**
- **temperature range 40-150 ℃**
- **processing times 5-60 min**

http://www.foodtechinfo.com/FoodPro/FacilityTypes/311222_Soybean_Processing.htm

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TF10 Protein Safety - New Issues

Examples: in vitro impact of heat on protein activity

Protein	***In vitro* heat**	**Activity**
CP4 EPSPS	65-75 °C, 30 min	none
CP4 EPSPS	soy toasted meal	none
CP4 EPSPS	soy protein isolate	none
mEPSPS	65 °C, 30 min	none
PAT	55 °C, 10 min	none
GAT	56 °C, 15 min	none
ALS	50 °C, 15 min	none
ß-glucuron.	60 °C, 15 min	50% loss
ß-amylase	bake 57-72 °C, 2 min	none
α-amylase	bake 68-83 °C, 4 min	none
Cry1Ab	80 °C, 10 min	none
C		
C		
C		

Conclusion: Dietary exposure to functionally active introduced proteins in processed food is likely negligible.

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TF10 Protein Safety - New Issues

For risk assessment, can the Threshold of Toxicological Concern (TTC) be applied to proteins?

Level of human intake or exposure that is considered to be of negligible risk despite the absence of chemical-specific toxicity data

Recommended for prioritizing risk from exposure to substances present at low levels in foods where toxicology data are limited

- **Proteins were excluded from development of a TTC because there is no agreement on a safe threshold for exposure to food toxins and allergens**
- **An introduced protein could be considered for TTC if it does not fit the profile of known toxins and allergens**
  - **digestible**
  - **low (ppm) levels in food**
  - **not structurally related to known toxins or allergens**

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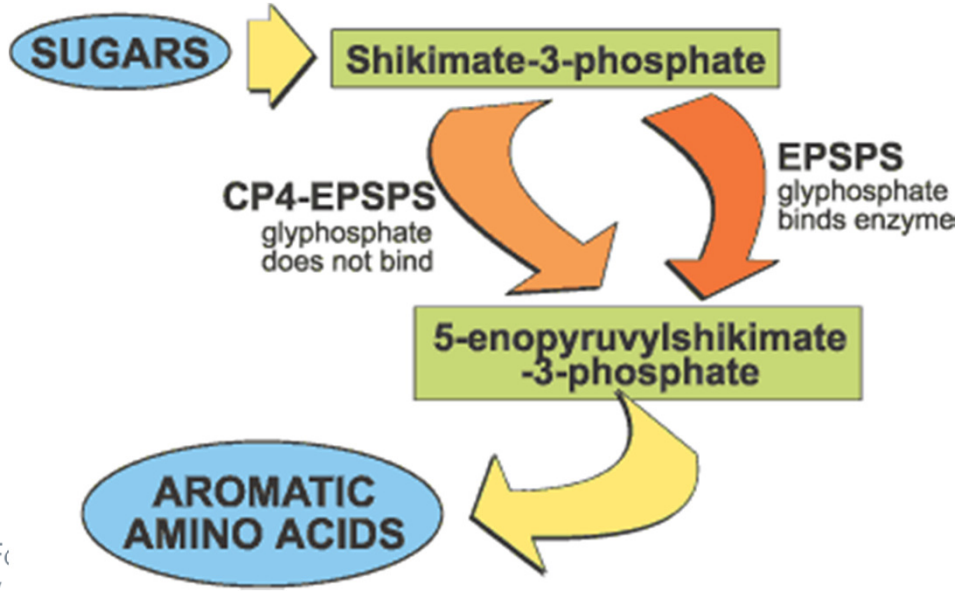
TF10 Protein Safety - New Issues

Example: TTC of CP4 EPSPS enzyme

5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)

- **enzyme from aromatic amino acid synthesis in plants and microbes**
- **variant from bacterial strain CP4 imparts tolerance to glyphosate herbicide**



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TF10 Protein Safety - New Issues

Example: TTC of CP4 EPSPS enzyme

- **Potential chronic intake of CP4 EPSPS from consumption of glyphosate tolerant maize was estimated to be 4 µg/kg/day**
- **This is 600× lower than TTC chronic limit, assuming no denaturation of CP4 EPSPS during processing of maize**
-
- **More realistic exposure – processing reduces CP4 EPSPS ~ 2 orders of magnitude – 60,000× lower than TTC chronic limit**

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New Issues Considered by TF10 Regarding Protein Safety

- What is the impact of food processing on potential dietary exposure to functionally active introduced proteins?
- For purposes of risk assessment, can the Threshold of Toxicological Concern (TTC) model be applied to proteins?
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TF10 Protein Safety - New Issues

What are the criteria for assessing "History of Safe Use" (HOSU)?

EFSA (2009) - acute toxicity studies with introduced proteins provide little value in the risk assessment of genetically engineered crops

However, EFSA will request 28-day repeat dose toxicology studies for proteins that do not have a HOSU unless there is reliable information demonstrating safety

- **Proteins with no HOSU are "novel"**
- **What constitutes "novel" with respect to introduced proteins?**

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Protein Evolution: What is Novel?

- **Average Mutation Rate**
- **1 base pair (bp) change in 1,000,000,000 bp for each generation**
- **a gene of 1,000 bp undergoes one mutation in 1,000,000 cell generations.**

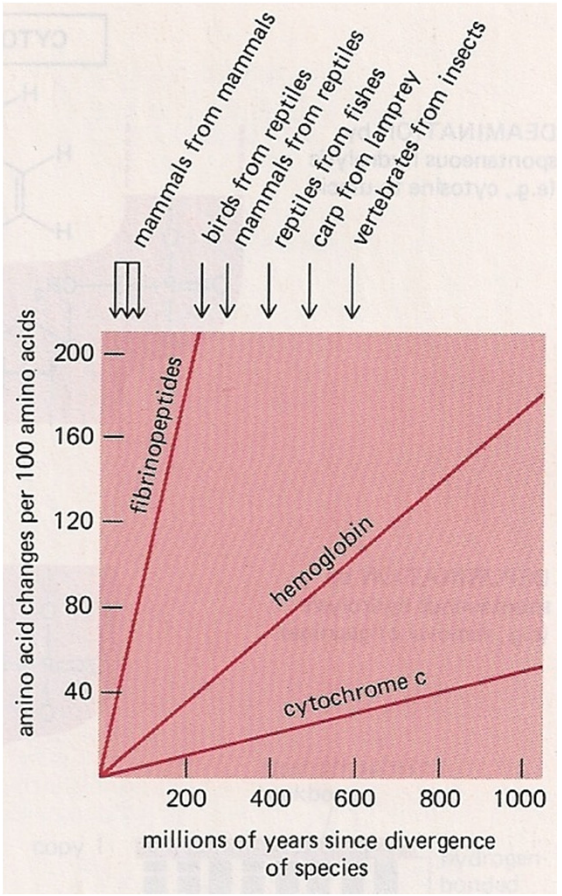
- **Most mutations are deleterious and eliminated by natural selection. Based on comparisons of homologous proteins across species, each protein shows a different but characteristic rate of evolution.**

cytochrome c: 6.7 changes per 100 amino acids every 100 million years
histone H4: 0.25 changes per 100 amino acids every 100 million years

- **Number of changes is much lower than expected for a spontaneous mutation rate because most changes are deleterious (i.e., negative consequences!)**

- **Same approach can be used to estimate the frequency of negative mutations**

hemoglobin: 6 out of 7 are harmful
cytochrome c: 29 out of 30 are harmful
histone H4: nearly any change is harmful



Wilson AC *et al.*, 1977 and 1987

Protein Evolution: Homologous Proteins

Example: EPSPS in plants and microbes

The amino acid content of EPSPS in soy, maize, and Baker's yeast varies considerably from CP4 EPSPS amino acid content

- **23 to 41% amino acid sequence identity**
- **49 to 59% amino acid sequence similarity**

Functionally related proteins can vary considerably in amino acid sequence yet maintain identical biological function through conserved active sites and tertiary structures

Consequently, the HOSU of related EPSPS found in foods could be considered as evidence for the safety of CP4 EPSPS. Prior to its introduction into food crops, there was no history of human consumption of CP4; however, homologous EPSPS are commonly found in foods.

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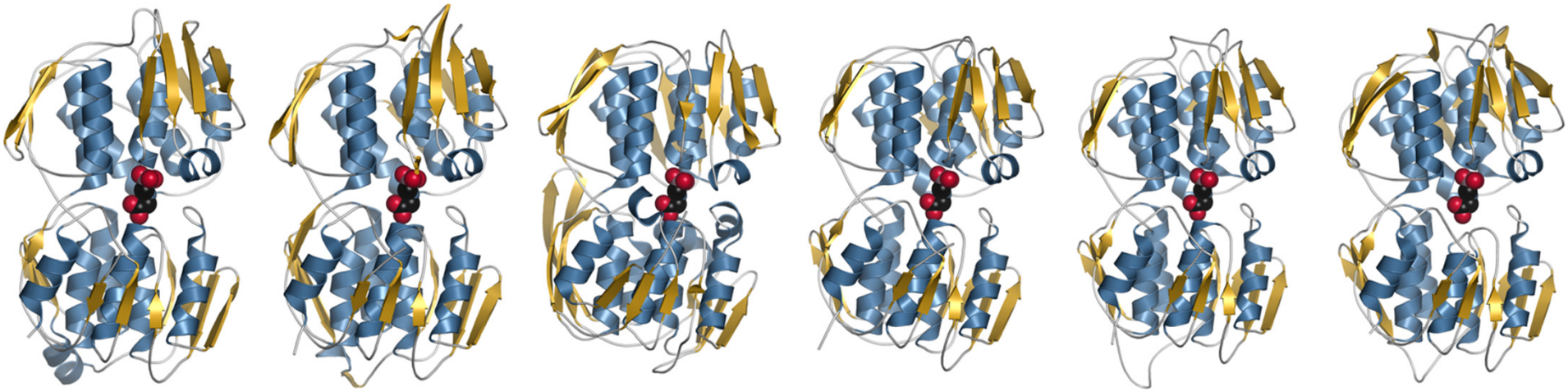
Protein Evolution: Homologous Proteins

Find the CP4 EPSPS?



sequence relationship: 27-90% identity with CP4 enzyme
conserved structure; invariant function

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TF10 Protein Safety - Summary

Toxicology studies would not be needed based on the following considerations:

- **If an introduced protein has no HOSU, but is structurally and functionally similar to those that do, its mode of action is likely to be similar (CP4 EPSPS and native EPSPS)**
- **Modifications in the primary structure of a non-toxic protein are not likely to make it toxic - requires molecular mechanism of action**
- **Proteins denature and lose function during food/feed processing.**
- **Human dietary exposure to functionally active introduced proteins in processed food fractions is likely to be low and poses a negligible risk (far below estimated TTC for proteins)**

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TF10 Protein Safety - Summary

Toxicology studies may be needed on a functionally active introduced protein if it is:

- **structurally or functionally related to known mammalian toxins**
- **stable in simulated gastric fluids**
- **stable to food processing conditions**
- **has a mode of action that raises a toxicological concern**
- **not sufficiently characterized regarding its mode of action and where there is a toxicological basis of concern**

When toxicology testing is considered necessary it should be hypothesis-driven and employ an appropriate study design and endpoints to address the hypothesis.

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TF10 Protein Safety - Summary

- **Toxicology testing of proteins introduced into genetically engineered crops has shown no evidence to date of adverse effects (acute or repeat dose)**

- **Introduced proteins are often variants of those found in nature**

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