JCTLM Members and Stakeholders Meeting
BIPM Sevres, France - 14 December 2004

# RECONCILING CLASSICAL BIOLOGICAL ASSAYS and METROLOGY-BASED BIOASSAYS

*A Challenge for Today*

# JCTLM Members and Stakeholders Meeting
# BIPM Sevres, France - 14 December 2004

## Relating Structure and Function in the Era of

## *Genomics*, *Proteomics*, *Metabolomics*

## and

## *Evidence-Based Medicine*

## *The Challenge for Tomorrow*

# CONTEMPORARY APPROACHES TO STANDARDIZATION OF BIOASSAYS

## WHO Standardization Approach

***Permits:***

- Heterogeneous reference material (unknown extent accepted)
- Multiple assay methods (parallel dose-response required)
- Average value assigned from multiple methods (functional and immunoassay results frequently assigned separate values)
- Unit is arbitrary (comparisons are problematic)
- Values considered to be “error free” (uncertainty unreported)
- Traceability (limited to prior reference material lot)

# *WHO* APPROACH – CLASSICAL BIOASSAY

***World Health Organization,*** **Technical Report Series No 800, p186-87**

## CRITICAL PRECEPTS:

*WHO* establishes international biological standards and reference reagents for substances of biological or synthetic origin that **cannot be characterized adequately by chemical and/or physical means alone** and that are used in the prophylaxis, therapy or diagnosis of human and certain animal diseases.

The purity of the material should be such that **no substances are present that would interfere with the procedures by which the material is to be tested**, but it should be noted that the purest material is not necessarily the most suitable. Less pure forms may be preferable if they are more stable or if the pure form is otherwise unsuitable.

# INHERENT LIMITATIONS OF THE *WHO* APPROACH

(Complexity of Biological Materials)

- **Analytes are heterogeneous and commonly uncharacterized**
  - Reference lots may differ in both the designated analyte and in the identities of the influence quantities
  - Reference lots may differ in the amounts of influence quantities and "matrix effects"
- **Influence quantities / matrix effects are undefined (indefinable?)**
- **Dose-response behavior is empirical**
  - Often nonlinear or linearized through data transforms
  - Different regions of the actual dose-response curves may be very susceptible to non-comparability of results

# REFERENCE SYSTEM APPROACH

**Uriano, GA and Cali, JP (1977)** Role of Reference Materials and Reference Methods in the Measurement Process in *Validation of the Measurement Process by* J.R. De Voe, ed., American Chemical Society, Washington, pp. 140-161

## What is meant by the *Measurement Process*?

- **A *scale* is required to quantitatively estimate the value of an intrinsic or extrinsic property of a material or system.**
- **An accurate method for applying the *scale* to whatever property is being measured is essential.**

Arbitrary units should be avoided, since it is necessary for making comparisons to have a common scale.

# REFERENCE SYSTEM APPROACH

**Uriano, GA and Cali, JP (1977)** Role of Reference Materials and Reference Methods in the Measurement Process in *Validation of the Measurement Process by* J.R. De Voe, ed., American Chemical Society, Washington, pp. 140-161

"**Reference materials and/or reference methods** are seen to be **two necessary but not always sufficient mechanisms** for achieving measurement compatibility between laboratories on a national scale."

"By using reference materials, **measurement compatibility can be achieved on the basis of precision alone,** if all parties agree to use the same measurement methods and reference material."

Point #2
Internationally – Clearly Impossible

# CONTEMPORARY APPROACHES TO STANDARDIZATION OF BIOASSAYS

## Metrological Reference System Approach

***Requires:***

- Reference materials ($1^{o}$ and $2^{o}$ distinguished in hierarchy)
- Validated reference measurement procedure
- Specified uncertainty
- Explicit description (influence quantities identified and corrections for systematic error / bias offered)
- Traceability to a higher metrological order

# LIMITATIONS INHERENT IN THE REFERENCE SYSTEM APPROACH

*Challenge of Macromolecules*

- **May not distinguish between functionally active and inactive molecules**
  - Molecules with Impaired Functionality
  - Molecular Heterogeneity
    - Multimeric structures (Site obstruction)
    - Molecular weight heterogeneities

# LIMITATIONS INHERENT IN THE REFERENCE SYSTEM APPROACH

## *Challenge of Macromolecules*

- **May not distinguish between functionally active and inactive molecules**
  - Molecules with Impaired Functionality
  - Molecular Heterogeneity
    - Multimeric structures
    - Molecular weight heterogeneities
- **USUALLY…only total concentrations are measured**
- **Relationships between reference procedures and routine procedures may not be readily established outside specialized reference measurement laboratories**

# Reconciling Concentration (Substance Amount)
## and
# Functional Activity Measurements
(*derived from USP definitions*)

***Start with concepts already in use***

- **Functional Activity** — *ability* of a substance to produce a defined ***biological effect***.
- **Potency** — measure of the biological activity using a suitable quantitative biological assay based upon an **attribute** which is linked to the relevant biological property.
- **Specific Activity** – arbitrary units divided by total mass

Reconciliation by linking attributes to function.

# A DEFINITION FOR FUNCTIONAL ACTIVITY

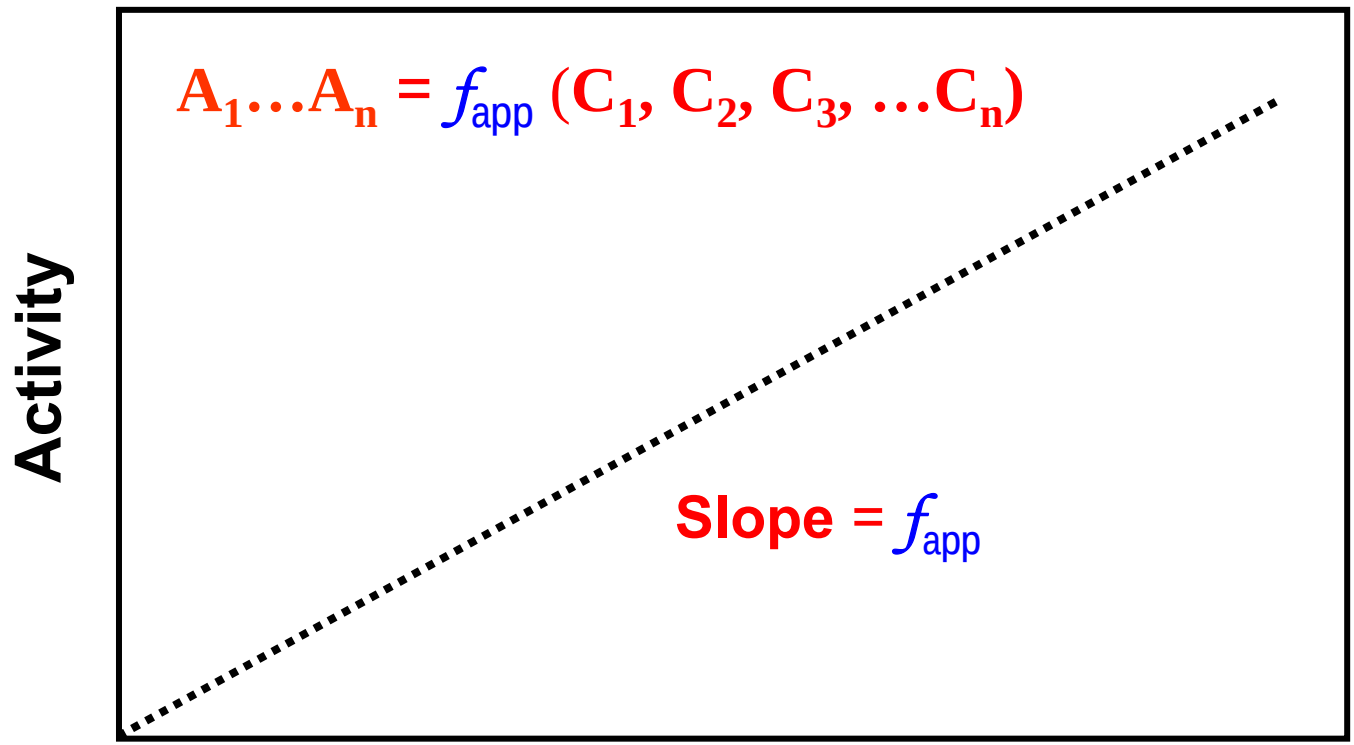
$$A = C \cdot f$$

- Functional activity **A**, is the product of two variables: the *concentration of active molecules,* **C** (mol•vol$^{-1}$), and a parameter $f$ (sec$^{-1}$ for time-dependent processes), which is independent of the concentration.

- Only the product of **C** and $f$ is measured unless the reference material is chemically and physically homogeneous and the concentration of functional molecules is independently known.

The measurement of functional activity has two parts that are generally not measured independently.

# THE SIMPLE EMPIRICAL SITUATION
# A LINEARIZED DOSE – RESPONSE CURVE
## *Dilutions of a reference material*



# Applying $A = C \cdot f$ to Enzymes

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_c} P + E$$

- **E** (an enzyme) has a binding site for **S** (substrate). The "affinity" of the site on **E** for **S** is characterized by a kinetic constant $K_m$.
- The chemical transformation of **S** into **P** is described by a second constant, $k_c$.
- The active site of the enzyme is the **ATTRIBUTE** of interest.

Functional activity in an enzyme-catalyzed reaction is the quantitative description of the enzyme's ability to convert a particular substrate into product and is related to the enzyme concentration by $f$.

# DEFINING FUNCTIONAL ACTIVITY QUALITATIVELY AND QUANTITATIVELY

- Functional activity is defined **qualitatively** by a chemical equation or a set of chemical equations that together describe the activity.

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_c} P + E$$

# DEFINING FUNCTIONAL ACTIVITY QUALITATIVELY AND QUANTITATIVELY

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$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_c} P + E$$

- Functional activity is **defined quantitatively** by the mathematical equation

$$A = C_E \cdot f_{E(S)}$$

# Interpreting $f$ for an Enzyme-Catalyzed Reaction

- In the simplest case, where **[S] << $K_m$** :

  $f_{E(S)}$ is the specificity constant ≡ $k_C / K_M$

  and $A = [S] \cdot f_{E(S)}$

- IF **[S]** is **≥ $K_m$** :

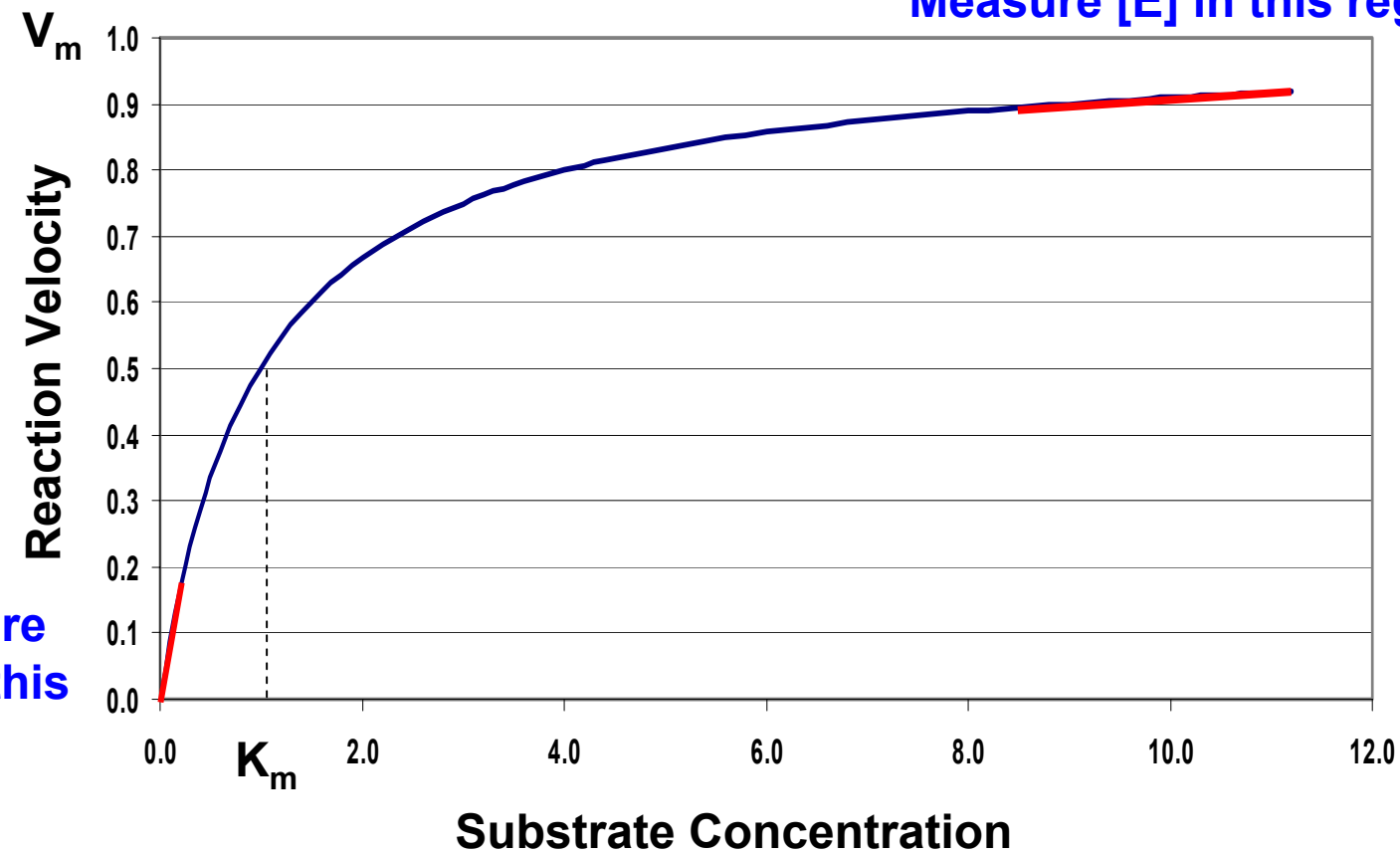
  $f_{E(S)}$ becomes ≡ $k_C [S] / (K_m + [S])$

For an enzyme-catalyzed reaction $f$ is simply described by the Michaelis-Menten equation, where $C$ is equivalent to [E].

# Applying $A = C \cdot f$ to Enzymes

## "Linear" and Non-linear Regions of Substrate Concentration Dependence

Measure [E] in this region




Measure [S] in this region

# WHERE DOES THE *katal* (SI unit) COME FROM?

The ***katal*** is equivalent to $\mathbf{A}$ times the volume (liters) and $f$ ($s^{-1}$) is a parameter that includes the effects of solution composition, pH, temperature, etc.

# HOW ARE INFLUENCE QUANTITIES HANDLED?

- **Influence quantities** are substances that may bind to an enzyme and alter its catalytic activity

OR

may be the reactants of a parallel reaction that produces the same measurand.

Influence quantities are defined using additional chemical equations.

# Applying $A = C \cdot f$ to Enzymes

## in the presence of a modifier i.e an *influence quantity*


**[M] = *Influence quantity concentration***

$$[E]_{total} = [E] + [EM]$$

$$A = C_E \cdot f_E + C_{E(M)} \cdot f_{E(M)}$$

The value of $f_{obs}$ depends on both the fraction of the free enzyme and the fraction of the enzyme to which the modifier is bound.

# DEFINE THE FIRST ATTRIBUTE

*Using the Michaelis-Menten equation*

**ACTIVE SITE is the ATTRIBUTE**


$$[E]_{total} = [E] + [ES]$$

$$A = C_E \cdot f_E$$

# DEFINE THE MODIFIER ATTRIBUTE

*Using a second simple chemical equation*

**MODIFIER Site as another ATTRIBUTE**

$$E + S \rightleftharpoons ES \longrightarrow E + P$$

$$+$$

$$M \rightleftharpoons EM + S \rightleftharpoons EMS \longrightarrow EM + P$$

$$[E]_{total} = [E] + [ES] + [EMS] + [EM]$$

$$A = C_E \cdot f_E + C_{E(M)} \cdot f_{E(M)}$$

# A KINETIC EQUATION FOR ENZYME ACTIVITY IN THE PRESENCE OF A MODIFIER

**Laidler, KJ & Bunting, PS (1973)** The Chemical Kinetics of Enzyme Action Oxford Univ. Press

**At a particular [S] and [M] :**

$$A = (k_c/K_m)_{obs} =$$

$$[\{(kc/K_m)_E + (k_c/K_m)_{EM}[M]/K_M\}[E_t]]/(1 + [M]/K_M)$$

$$f_E = (k_c/K_m)_E$$

$$f_{E(M)} = (k_c/K_m)_{E(M)}$$

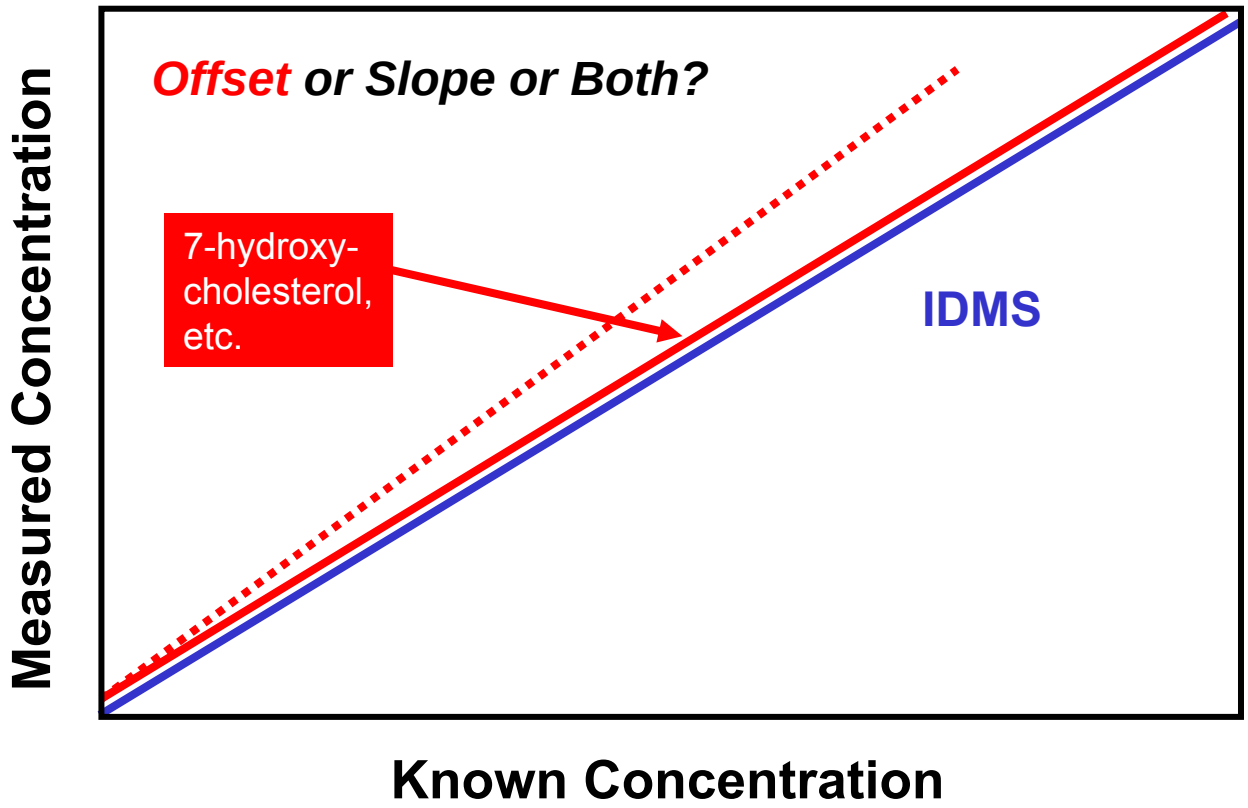
# $f$ IN OTHER TYPES OF REACTIONS

- Empirical Bioassay Reactions $A = C_{app} \cdot f_{app}$
- Modulated Enzyme Reactions $A = C_E \cdot f_E + C_{E(M)} \cdot f_{E(M)}$
- Parallel Reactions (two enzymes) $A = C_1 \cdot f_1 + C_2 \cdot f_2$
- Sequential Reactions (multiple enzymes)
- Methods for Metabolites – Routine and Reference

  Glucose (hexokinase, glucose oxidase) $f$ may be equal to unity

  Cholesterol (Abell-Kendall) $f$ may differ from IDMS

# CHOLESTEROL
## REFERENCE MEASUREMENT PROCEDURES
### Abell Kendall vs Isotope Dilution Mass Spectrometry




**Ref:** Bernert, J.T., et al. Clin Chem 37: 2053-2061 (1991)

# APPLYING THE DEFINITION $A = C \cdot f$ TO MACROMOLECULES

While the equation for Functional Activity might appear to be too simple for biological molecules that have multiple sites and attributes,

it is straightforward to define an $f$ for each attribute of the macromolecule.

# Applying $A = C \cdot f$

## Binding to Macromolecules

**DEFINING ATTRIBUTES:**

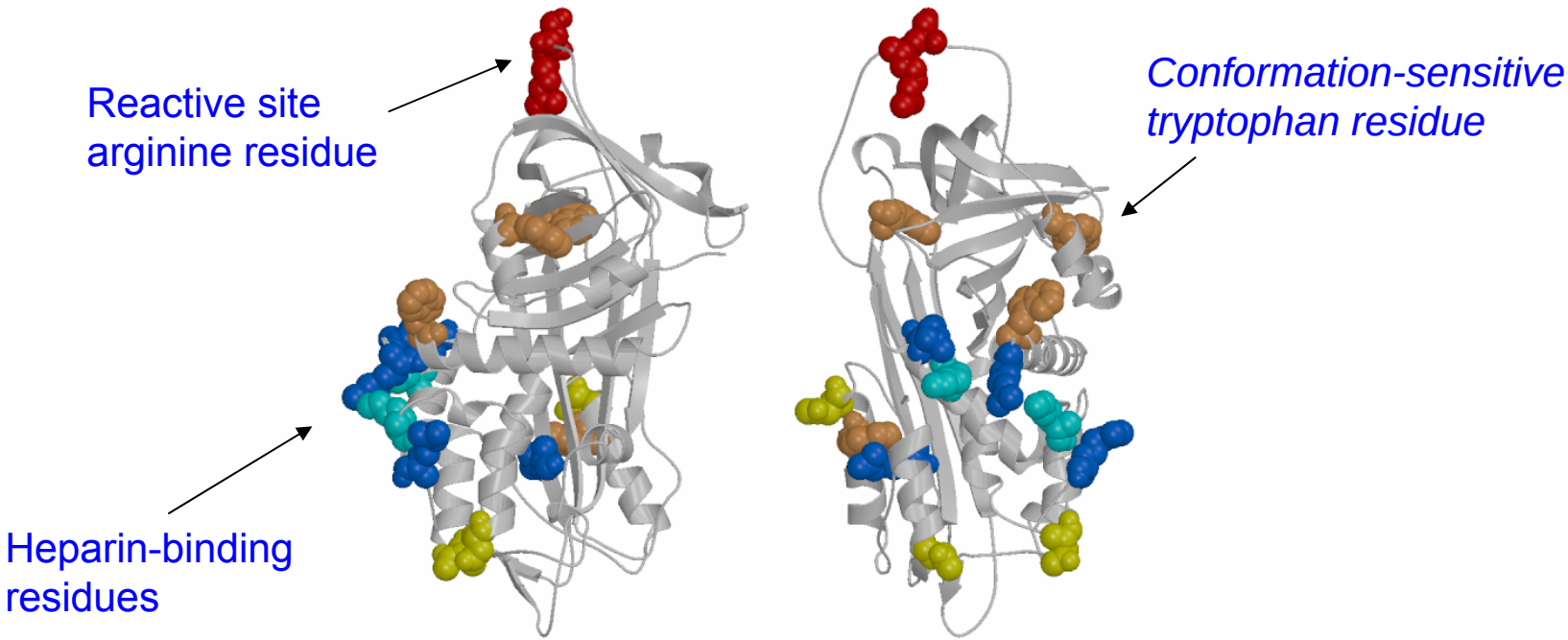
- Each function is the property of a single **attribute.**
- Each function is defined by a chemical equation that describes the function associated with the **attribute** .

**In blood coagulation reactions several attributes may be necessary to account for the "normal" physiological process.**

# EXPANSION OF THE DEFINITION $A = C \cdot f$

## Applying $f$ to ANTITHROMBIN

Define $f$ for each **ATTRIBUTE** of the macromolecule, not for the entire antithrombin molecule: $f_{AT(Attribute)}$




**ANTITHROMBIN (human)**

# Applying $A = C \cdot f$ to Macromolecules

## BINDING TO A SINGLE SITE ON A PROTEIN MOLECULE

$$L + P \underset{}{\overset{K_d}{\rightleftharpoons}} LP$$

$K_d$ in mol/L

- **P** (protein) has a binding site for **L** (ligand)
- The affinity of **P** for **L** is characterized by a dissociation constant) $K_d$.
- The **intrinsic functional competency** of **P** for binding **L** is described by $f_{P(L)}$.
- The concentration of **LP** is determined by the concentration of **L** and $K_d$, where **[LP] = [L][P] / $K_d$**

In a binding process, $f_{P(L)}$ is equivalent to the affinity

# General Applications

- Enzyme-based Assays
  - Enzyme Activity
  - Substrates
  - Modifiers
- Binding Assays
  - Receptors
  - Agonists
  - Antagonists
- Immunoassays
- Nucleic Acid Assays

# Collaborators in Development of the Approach

- Craig M. Jackson, Ph.D. San Diego, CA, USA
- M. Peter Esnouf, D.Phil., Oxford, UK
- Frederick A. Dombrose, Ph.D., Charlotte, NC, USA

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*Lothar Siekmann, Ph.D., Bonn, Germany*
*Brian A. Jackson, Ph.D., Springfield, VA, USA*

# References Upon Which the Presenter Has Drawn


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Thank You

# Functional Activity of a Mutant Enzyme

$A_{wt} = C_{wt} \cdot f_{wt}$ and $A_{mut} = C_{mut} \cdot f_{mut}$