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[54] **CHARGE EFFECTS IN ENZYME IMMUNOASSAYS**

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[58] **Field of Search** ..... 435/4, 7, 177, 188, 435/805, 810, 5; 424/8, 12, 85; 260/112, 121; 252/408 R; 73/230 B; 422/8

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4,104,029	8/1978	Maier et al.	435/188
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[57] **ABSTRACT**

A method for determining a member of a specific binding pair-ligand and receptor (antiligand). Reagents employed include a first modified member which provides an electrical field due to the presence of a plurality of ionic charges and a second modified member labeled with a component of a signal producing system, which system may have one or more components. The average proximity in the assay medium of the first and second modified members is related to the amount of analyte, where the observed signal from the signal producing system is related to the effect of the electrical field on the signal producing system.

Also, compositions are provided, as well as reagents, in predetermined ratios for optimizing the signal response to variations in analyte concentration.

15 Claims, No Drawings

CHARGE EFFECTS IN ENZYME IMMUNOASSAYS

BACKGROUND OF THE INVENTION

1. Field of the Invention

Protein binding assays or immunoassays have been the subject of thorough investigation and commercialization. The ability to specifically determine a drug or other compound of interest, particularly physiological interest, which is present in extremely low concentration, usually less than micrograms per milliliter, has opened up new opportunities in clinical laboratories. The ability to monitor therapeutic drug administration or drug addiction, to rapidly and efficiently determine diseased states, and to monitor the condition of a patient during times of stress, has provided significant opportunities for improvement of health care.

The assays in the clinical laboratory frequently require not only high sensitivity but accuracy over a relatively narrow range. Therefore, new techniques are being developed which recognize these requirements by providing for greater sensitivity, reduced response to non-specific effects, and easier and simplier protocols. In addition, many antigens can be obtained only in impure form or in pure form at elevated costs. Therefore, assays must accomodate the possibility that the antigen will be impure. Parallel to this situation is that most antibodies which are obtained by antigenic injection will have less than about 30 weight %, or frequently less than about 20 weight % of the total protein as the antibody of interest. When preparing reagents which involve reactions with the antibody composition, the presence of the large amount of contaminant must be taken into account.

Other considerations in developing an assay include the necessity for and number of incubations, the period required for the incubation, the period required for the measurement, the sensitivity of the measurement to extraneous factors, the stability of the reagents, the formulation of the reagents, and the like.

2. Description of the Prior Art

U.S. Pat. No. 3,996,345 describes the employment of a chromophore pair—fluorescer and quencher—, where the members of the pair are bonded to different members of a specific binding pair, so that the amount of fluorescer and quencher which come within an interacting distance is dependent upon the amount of analyte in the medium. U.S. Pat. No. 3,935,074 describes an immunoassay dependent upon the inability of two antibodies to simultaneously bind to a reagent having at least two determinant sites. Co-pending application Ser. No. 893,650, filed Apr. 5, 1978, U.S. Pat. No. 4,233,402, teaches the concept in immunoassays of bringing together two enzymes, whose substrates or products are in some ways related to the production of a detectible signal, where the juxtaposition of the enzymes is related to the amount of analyte in the medium. Co-pending application Ser. No. 815,632, filed July 14, 1977, U.S. Pat. No. 4,208,479, teaches the employment of a macromolecular modifier of a label bound to an antibody, where the modifier is inhibited from approaching the label when the labeled antibody is bound to antigen. Co-pending application Ser. No. 815,487, filed July 14, 1977, U.S. Pat. No. 4,233,401, discloses an enzyme immunoassay where ligand is labeled with enzyme and an enzyme inhibitor is inhibited from approaching the enzyme, when antibody is bound to the ligand. Co-

pending application Ser. No. 964,099, filed Nov. 24, 1978, discloses the use of macromolecular particles to provide discrimination between a label bound to the particle and a label free in the solution, where the amount of label bound to the particle is related to the amount of analyte in the medium, and the observed detectible signal is dependent upon the distribution of the label between the particle and the medium. U.S. Pat. No. 3,817,837 describes a homogeneous enzyme immunoassay.

SUMMARY OF THE INVENTION

A protein binding assay is provided involving members of a specific binding pair, the members being ligand and receptor (antiligand). As a first reagent in the method, one of the members is substituted with a plurality of ionizable functionalities which are capable of providing a charge, either positive or negative, under the assay conditions. A second member, either the same or homologous member, is labeled with a component of a signal producing system, which signal producing system is able to produce a detectible signal under the assay conditions.

The signal produced by the signal producing system is affected by the juxtaposition of the electrical field resulting from the charged first member. By appropriate choice of the members in relation to the analyte, the average proximity of the first and second members in the assay medium can be related to the amount of analyte in the assay medium. Thus, the observed signal can be related to the amount of analyte in the assay medium. By employing appropriate standards having known amounts of analyte, one can establish a relationship between concentration of the analyte and the level of observed signal.

In addition, reagents are provided for the assay, as well as combinations of reagents which provide for substantial optimization of the sensitivity of the assay or level of response of the assay to variations in the concentration of the analyte.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The subject invention is concerned with a protein binding assay, involving a specific binding pair, ligand and receptor. The basis of the invention is to relate the proximity of two reagents to the amount of analyte in the assay medium. The first reagent, is a first member of the specific binding pair which is modified with a plurality of ionic charges, so as to create a charged field in an aqueous environment at a predetermined pH. A second member is modified with a label, which is one component of a single or multicomponent signal producing system; the level of the observed signal is dependent upon the juxtaposition of the signal producing system to the field created by the charged member. The field affects the signal producing system by enhancing or diminishing the localized concentration of certain ions in the assay medium, which ionic concentration affects the level of the observed signal. In addition to the analyte and the first and second members, other materials may also be added depending upon the nature of the signal producing system.

The binding of homologous members of the specific binding pair results in the formation of a complex, where two or more, usually three or more members are involved. In view of the polyvalent nature of antibodies

and antigens, the complex can be extended to create a network of antibody and antigen bringing a plurality of signal labels and ionic charges into proximity, where interactions can occur.

In discussing the subject invention, the following order will be involved. First, the assay method will be considered. This will be followed by definitions defining various terms in relation to the materials employed. Following the definitions, the materials will be discussed as to the analyte, signal producing system, and charged member. This will be followed by the experimental and demonstration of the utility of the subject method.

ASSAY METHOD

In accordance with the subject method, the analyte, a reagent having a plurality of ionizable groups which are substantially ionized under the conditions of the assay, a labeled reagent, where the label is a member of a signal producing system and any necessary additional components are combined in a buffered aqueous medium. The observed signal may then be read and compared to an assay medium having a known amount of analyte.

The analyte is a member of a specific binding pair, consisting of ligand and its homologous receptor, and either the ligand or the receptor may be the analyte. The assay medium is normally aqueous, which is normally buffered in sufficient amount to a moderate pH, generally close to providing optimum assay sensitivity. The assay is performed without separation of the assay components or products.

The aqueous medium may be solely water, or may include from zero to 40 volume percent of a cosolvent, normally a polar solvent, usually an oxygenated organic solvent or from one to six, more usually of from one to four carbon atoms, including alcohols, ethers, and the like.

The pH for the medium will usually be in the range of about 4 to 11, more usually in the range of about 5 to 10, and preferably in the range of about 6.5 to 9.5. The pH is chosen so as to maintain a significant level of specific binding by the receptor, while optimizing the sensitivity or response of the signal producing system to variations in analyte concentration.

Various buffers may be used to achieve the desired pH and maintain the pH during the determination. Illustrative buffers include borate, phosphate, carbonate, tris, barbital and the like. The particular buffer employed is not critical to this invention, but in individual assays, one or another buffer may be preferred.

Moderate temperatures are normally employed for carrying out the assay and usually constant temperatures during the period of the measurement, particularly for rate determinations. Incubation temperatures will normally range from about 5° to 45° C., more usually from about 15° to 40° C. Temperatures during measurements will generally range from about 10° to 50° C., more usually from about 15° to 40° C.

The concentration of analyte which may be assayed will generally vary from about 10^{-4} to 10^{-15} M, more usually from about 10^{-6} to 10^{-13} M. Considerations, such as whether the assay is qualitative, semiquantitative or quantitative, the particular detection technique and the concentration of the analyte of interest, will normally determine the concentrations of the other reagents.

While the concentrations of the various reagents in the assay medium will generally be determined by the concentration range of interest of the analyte, the final concentration of each of the reagents will normally be determined empirically to optimize the sensitivity of the assay over the range of interest. That is, a variation in concentration of the analyte which is of significance should provide an accurately measurable signal difference.

The total binding sites of the members of the specific binding pair which are reciprocal to the analyte will vary widely depending upon the nature of the reagents, that is, whether the reagents are the same as or different from the analyte. For example, both reagents may be receptors or both reagents may be ligand or one of the reagents may be ligand and the other reagent receptor. At least one reagent will be the receprocal bonding agent of the analyte.

The ratio of labeled reagent to analyte based on binding sites in the assay medium will generally be from about 0.5 to about 100 binding sites of labeled reagent per binding site of analyte, usually from about 1 to 50, and more usually from about 1 to 20 over the analyte concentration range. These members refer to available sites at saturation since all sites will not be equally available.

It should be appreciated that these numbers are merely intended to be illustrative of the ratios of most likely interest. The ratio will vary depending upon the manner of measurement, equilibrium or rate, the binding constant of the labeled reagent, the concentration of the analyte, the sensitivity of the signal producing system to charge effects, the nature of the ligand, and the sensitivity with which the signal may be detected.

The mole ratio of the charged member to the labeled member may also be varied widely, depending upon the nature of the label, the sensitivity of the label to charge effects, and the nature of the ligand. Usually, the mole ratio of the charged member to the labeled member will be from about 0.5–100:1, more usually from about 1 to 20:1. This can vary quite dramatically, depending upon the particular protocol, the order of addition, the nature of the label, the relative binding affinities and the like.

The order of addition may vary widely, although frequently all of the components of a multicomponent signal producing system will not be added simultaneously. Usually, the member labeled with a component of the signal producing system will be added to the assay medium prior to at least one of the other components of the signal producing system. This will be particularly true, where an enzyme is a label and the other components are substrates and cofactors.

The two reagent members, may be added simultaneously or consecutively to the analyte. Conveniently, the signal producing system label will frequently be added prior to the charged member. The two reagents may be provided as a single composition or as separate compositions, depending upon the nature of the protocol.

Two particular protocols may be indicated as illustrative. The first protocol involves the addition of the signal producing system labeled member to the analyte and incubating for a sufficient time for the system to at least approach equilibrium. To the mixture may then be added the charged member and at the same time or immediately thereafter, any additional components of the signal producing system.

An alternative protocol would be to add substantially simultaneously, the signal producing system labeled member and the charged member and either incubate or not, as required. Desirably, one may add a signal inhibi-

tor; that is, a material which interacts with the signal producing system label, so as to inhibit production of the signal, when the signal producing system labeled member is not bound to its homologous member.

The analyte may act to bring the charged member and the signal label member together or enhance the separation of the charged and signal label members. For example, when the analyte is an antigen or poly(ligand analog) and the two reagent members are antibodies, within a limited concentration range, the analyte will serve to bring on the average the two reagent members in closer proximity than when the members are diffusing freely in solution. On the other hand, when the analyte is an antigen and the signal label member is an antigen, then the analyte and signal label member will compete for a charged antibody.

One or more incubation steps may be involved in preparing the assay medium. For example, it may be desirable to incubate an antigen analyte with labeled receptor. In addition, it may be desirable to have a second incubation step, depending upon the nature of the other reagents employed. Whether to employ an incubation period and the length of the incubation period will depend to a substantial degree on the mode of determination—rate or equilibrium—and the rate of binding of the receptor to the ligand. Usually, the time for incubation steps will vary from about 0.5 min to 6 hrs, more usually from about 5 min to 1 hr. Incubation temperatures will generally range from about 4° to 50° C., more usually from about 15° to 37° C.

After the reagents are combined, the signal will then be determined. The method of determination will normally be the observation of electromagnetic radiation, particularly, ultraviolet and visible light, more particularly visible light, either absorption or emission. Desirably, where fluorescence is involved, the light emitted should have a wavelength in excess of 400 nm, more desirably in excess of 450 nm, and preferably in excess of 500 nm. Where absorption is involved, the absorption will normally be in the range of about 250 to 900 nm, more usually from about 325 to 650 nm.

The temperature at which the signal is observed will generally range from about 10° to 50° C., more usually from about 15° to 40° C.

Standard assay media can be prepared which have known amounts of the analyte. The observed signal for the standard assay media may then be plotted so as to relate concentration to signal. Once a standard curve has been established, a signal may be directly related to the concentration of the analyte.

The time for measuring the signal will vary depending on whether a rate or equilibrium mode is used, the sensitivity required, the nature of the signal producing system and the like. For a rate mode, the times between readings will generally vary from about 5 sec to 6 hrs, usually about 10 sec to 1 hr. For the equilibrium mode, after a steady state is achieved, a single reading may be sufficient or two readings over any convenient time interval may suffice.

DEFINITIONS

Analyte—the compound or composition to be measured, which may be a ligand, a single or plurality of compounds which share at least one common epitopic or determinant site, or a receptor.

Specific binding pair—two different molecules, where one of the molecules has an area on the surface or in a cavity which specifically binds to a particular spatial and polar organization of the other molecule. The members of the specific binding pair are referred to as ligand and receptor (antiligand).

Ligand—any organic compound for which a receptor naturally exists or can be prepared.

Receptor (antiligand)—any compound or composition capable of recognizing a particular spatial and polar organization of a molecule i.e. determinant or epitopic site. Illustrative receptors include naturally occurring receptors, e.g. thyroxine binding globulin, antibodies, Fab fragments, enzymes, lectins and the like.

Ligand Analog—a modified ligand which can compete with the analogous ligand for a receptor, the modification providing means to join the ligand analog to another molecule. Depending upon the available functionalities on the ligand, the ligand analog may differ from the ligand by more than replacement of a hydrogen with a bond which links the ligand analog to a hub or label.

Poly(ligand-analog)—a plurality of ligand analogs joined together covalently, frequently to a hub nucleus. The hub nucleus is a polyfunctional material, normally polymeric, usually having a plurality of functional groups e.g. hydroxy, amino, mercapto, ethylenic, etc. as sites for linking. The hub nucleus may be water soluble or insoluble, usually water soluble, and will normally be at least about 10,000 molecular weight, usually at least about 35,000 molecular weight and may be 10 million or more molecular weight, usually under 600,000, more usually under 300,000 molecular weight. Illustrative hub nuclei include polysaccharides, polypeptides, including proteins, nucleic acids, ion exchange resins and the like. Water insoluble hub nuclei can include glasses, addition and condensation polymers, both cross-linked and non-cross-linked, naturally occurring particles, or the like, either having a plurality of functionalities or capable of functionalization.

Signal Producing System—the signal producing system may have one or more components, there being one component conjugated to a specific binding pair member. The signal producing system produces a measurable signal which is detectible by external means, normally the measurement of electromagnetic radiation. In the subject invention, the level of observed signal produced by the signal producing system will be susceptible to the proximity of the charged member to the labeled member. For the most part, the signal producing system will involve enzymes and chromophores, where chromophores include dyes which absorb light in the ultraviolet or visible region, phosphors, fluorescers and chemiluminescers. The enzymes will normally involve either the formation or destruction of a substance which absorbs light in the ultraviolet or visible region or the direct or indirect production of emitted light by fluorescence or chemiluminescence.

Label—any molecule conjugated to another molecule, particularly, the former being a member of the signal producing system. In the subject invention, the labels will be the signal producing system component bound to a member of the specific binding pair and will be referred to as the signal label.

Labeled Ligand—the conjugate of the ligand member (ligand analog) of the specific binding pair with a member of the signal producing system, either covalently or non-covalently bound, when covalently bound, either bound by a bond, linking group or hub nucleus. The labeled ligand may have one or more ligands or one or

more labels or a plurality of both, the latter being referred to as poly(ligand analog)-polylabel.

Labeled Receptor—the conjugate of receptor with a member of the signal producing system, where the two are bound either covalently or noncovalently, usually covalently by a linking group, where there may be one or more receptors bound to the label or one or more labels bound to the receptor.

Charged Member—a soluble member of the specific binding pair, usually the receptor, more usually antibody, being polyionic by being either covalently or non-covalently substituted with a plurality of functionalities of the same charge, either negative or positive, so as to create a relatively high localized density of a particular charge, which may be a single member or a plurality of members linked together.

MATERIALS

The components employed in the subject assay will be the analyte, which is a member of the specific binding pair, the signal labeled member, any additional components of the signal producing system, the charged member, and as appropriate receptor or poly(ligand analog).

Analyte

The ligand analytes of this invention are characterized by being monoepitopic or polyepitopic. The polyepitopic ligand analytes will normally be poly(amino acids) i.e. polypeptides and proteins, polysaccharides, nucleic acids, and combinations thereof. Such combinations of assemblages include bacteria, viruses, chromosomes, genes, mitochondria, nuclei, cell membranes, and the like.

For the most part, the polyepitopic ligand analytes employed in the subject invention will have a molecular weight of at least about 5,000, more usually at least about 10,000. In the poly(amino acid) category, the poly(amino acids) of interest will generally be from about 5,000 to 5,000,000 molecular weight, more usually from about 20,000 to 1,000,000 molecular weight; among the hormones of interest, the molecular weights will usually range from about 5,000 to 60,000 molecular weight.

The wide variety of proteins may be considered as to the family of proteins having similar structural features, proteins having particular biological functions, proteins related to specific microorganisms, particularly disease causing microorganisms, etc.

The following are classes of proteins related by structure:

- protamines
- histones
- albumins
- globulins
- scleroproteins
- phosphoproteins
- mucoproteins
- chromoproteins
- lipoproteins
- nucleoproteins
- glycoproteins
- proteoglycans
- unclassified proteins, e.g. somatotropin, prolactin, insulin, pepsin

A number of proteins found in the human plasma are important clinically and include:

- Prealbumin
- Albumin
- α_1-Lipoprotein
- α_1-Acid glycoprotein
- α_1-Antitrypsin
- α_1-Glycoprotein
- Transcortin
- 4.6S-Postalbumin
- Tryptophan-poor α_1-glycoprotein
- $\alpha_1\chi$-Glycoprotein
- Thyroxin-binding globulin
- Inter-α-trypsin-inhibitor
- Gc-globulin
  - (Gc 1-1)
  - (Gc 2-1)
  - (Gc 2-2)
- Haptoglobin
  - (Hp 1-1)
  - (Hp 2-1)
  - (Hp 2-2)
- Ceruloplasmin
- Cholinesterase
- α_2-Lipoprotein(s)
- Myoglobin
- C-Reactive Protein
- α_2-Macroglobulin
- α_2-HS-glycoprotein
- Zn-α_2-glycoprotein
- α_2-Neuramino-glycoprotein
- Erythropoietin
- β-lipoprotein
- Transferrin
- Hemopexin
- Fibrinogen
- Plasminogen
- β_2-glycoprotein I
- β_2-glycoprotein II
- Immunoglobulin G (IgG) or γG-globulin
  - Mol. formula: $\gamma_2\kappa_2$ or $\gamma_2\lambda_2$
- Immunoglobulin A (IgA) or γA-globulin
  - Mol. formula: $(\alpha_2\kappa_2)^n$ or $(\alpha_2\lambda_2)^n$
- Immunoglobulin M (IgM) or γM-globulin
  - Mol. formula: $(\mu_2\kappa_2)^5$ or $(\mu_2\lambda_2)^5$
- Immunoglobulin D(IgD) or γD-Globulin (γD)

VII	Proconvertin
VIII	Antihemophilic globulin (AHG)
IX	Christmas factor, plasma thromboplastin component (PTC)
X	Stuart-Prower factor, autoprothrombin III
XI	Plasma thromboplastin antecedent (PTA)
XII	Hagemann factor
XIII	Fibrin-stabilizing factor

Important protein hormones include:

Peptide and Protein Hormones

- Parathyroid hormone (parathromone)
- Thyrocalcitonin

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