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[54] **ASSAY EMPLOYING A LABELED FAB-FRAGMENT LIGAND COMPLEX**

[75] Inventor: **Moshe Schwarzberg**, Sunnyvale, Calif.

[73] Assignee: **Syva Company**, Palo Alto, Calif.

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[58] **Field of Search** ..... 424/1, 8, 12, 13; 250/302; 23/230 B; 435/7

[56] **References Cited**

U.S. PATENT DOCUMENTS

3,935,074	1/1976	Rubenstein	195/103.5
3,996,345	12/1976	Ullman	424/12
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Carrico, et al., Anal. Biochem. vol. 72, 1972 pp. 271–282, 283–292.
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Primary Examiner—Anna P. Fagelson
Attorney, Agent, or Firm—Bertram I. Rowland

[57] **ABSTRACT**

Methods and compositions are provided for improved protein binding assays by preparing compositions having indirectly labeled ligands substantially free of label conjugated to materials other than the indirectly labeled ligand. The method for preparing the compositions involves employing a monovalent receptor to which the label is conjugated and combining the monovalent receptor labeled conjugate to the ligand, either in pure or impure form. The mixture is then segregated according to molecular weight and the ligand conjugated to the labeled receptor isolated. This conjugate may then be directly used as a reagent in a protein binding assay, where the assay mixture is substantially free of label other than labeled receptor bound to ligand.

The labels will be for the most part of relatively low molecular weight, while the receptor is preferably a Fab fragment. The ratio of receptor to ligand will be chosen so as to provide reasonable molecular weight distinctions between unbound ligand, unbound labeled receptor, ligand bound to labeled receptor, and other materials which may be in the mixture. Various techniques may be used for the separation.

The assays are performed in accordance with known methods employing a second receptor composition, which may be labeled or unlabeled.

10 Claims, No Drawings

ASSAY EMPLOYING A LABELED FAB-FRAGMENT LIGAND COMPLEX

BACKGROUND OF THE INVENTION

1. Field of the Invention

The availability of molecules which are able to specifically bind to a particular spatial and polar organization is the basis for a wide variety of techniques referred to as competitive protein binding assays. These techniques depend upon having a member of a specific binding pair conjugated with the label which is involved with the production of a detectible signal.

Depending upon the nature of the label, various methods have evolved for distinguishing between an analyte which is bound to the corresponding member of the specific binding pair and analyte which is unbound. With the various techniques, either the receptor or the ligand is labeled. Particularly, where the receptor is labeled, the receptor is normally one component of a complex mixture of analogous composition and molecular weight. For example, antibodies which are isolated from serum will be present with globulins and other antibodies which are either non-specific or specific for a wide variety of ligands other than the ligand of interest. When labeling the receptor composition, both the receptor of interest as well as the contaminating globulins will be labeled. The label involved with extraneous receptors or other materials will act as a background in the assay, interfering with the sensitivity of the assay.

While affinity chromatography may be employed to enhance the purity of the receptor of interest, this technique has many deficiencies. One deficiency is that the most strongly binding antibodies tend to be retained by the affinity chromatography column. Secondly, there are normally substantial losses of the antibodies of interest and substantial reduction in the binding constant of the recovered antibody. It is therefore desirable to find alternative methods to provide labeled reagents having reduced amounts of label bound to extraneous materials.

2. Brief Description of the Prior Art

U.S. Pat. No. 3,998,943 discloses a fluorescent immunoassay involving a ligand conjugated to a fluorescer, employing receptor to ligand and receptor to fluorescer, where the receptor to fluorescer is inhibited from binding to fluorescer when receptor to ligand is bound to ligand. U.S. Pat. No. 3,935,074 describes an immunoassay where a receptor for a detector label and a receptor for ligand are employed with the detector label labeled to ligand. Various labels are described. U.S. Pat. No. 3,996,345 describes an assay employing a chromogenic pair, where one of the chromogens fluoresces emitting light at a wavelength within the absorption band of the other chromogen. Copending patent application Ser. No. 815,636, filed July 14, 1977 now U.S. Pat. No. 4,160,145, discloses the use of a non-enzymatic catalyst as a label in competitive protein binding assays.

Co-pending application Ser. No. 893,910, filed Apr. 5, 1978, describes a chemiluminescent label in a competitive protein binding assay. Assays dependent upon the presence of enzyme labile bonds are described in Carrico, et al, Anal. Biochem. 72, 271–282 (1976); ibid 72, 283–292 (1972).

SUMMARY OF THE INVENTION

Methods and compositions are provided for protein binding assays. The method of preparing reagents involves conjugating a relatively low molecular weight label to a monovalent receptor for a polyepitopic ligand, the ligand and receptor being members of a specific binding pair. The composition is then purified by separating the mixture according to molecular weight, so as to isolate the ligand bound to labeled receptor substantially free of other labeled compounds.

The resulting purified ligand bound to labeled monovalent receptor (labeled ligand) may then be employed as a reagent in a protein binding assay, where the presence of labeled or unlabeled polyvalent e.g. antibody, receptor bound to the labeled ligand affects the detectible signal produced by the label. Illustrative labels include fluorescers, chemiluminescers, nonenzymatic catalysts, groups having enzyme labile bonds, and the like.

Compositions are provided having labeled Fab antibodies bound to a polyepitopic ligand in the substantial absence of other labeled materials, as well as the label. These labeled ligand compositions are provided in kits with ligand receptor in premeasured amounts for use in protein binding assays.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The subject invention is concerned with novel compositions for use in protein binding assays, methods for preparing such compositions, combinations of reagents for use in protein binding assays, and improved methods for performing protein binding assays by employing the reagents of this invention.

The reagents of this invention are prepared by conjugating a relatively low molecular weight label capable of providing a detectible signal to a monovalent receptor, where the receptor is one component of a mixture of components having analogous chemical properties. The labeled mixture, preferably segregated as to molecular weight, is then combined with a ligand which will bind solely to its reciprocal receptor. The mixture is then subjected to segregation by molecular weight, segregating and isolating those fractions involving one or more of the monovalent labeled receptor bound to ligand. The resulting product is then substantially free of other materials bound to label, as well as the label, and also unbound ligand. Since these compositions are substantially free of label which is uninvolved with the assay, as well as other interferants, the observed signal is solely derived from the label which is bound to ligand through the monovalent receptor. Thus, the background which would result from label unrelated to ligand and its reciprocal receptor is substantially diminished or absent.

The labeled receptor-ligand complex may then be employed in a protein binding assay, the technique depending upon the particular label. The complex may be provided as a reagent in a kit in combination with receptor for ligand, where the two reagents are premeasured so as to substantially optimize the sensitivity of the assay.

Definitions

Analyte—the compound or composition to be measured, which may be a polyepitopic ligand, normally antigenic, having at least two epitopic sites, a mixture of

compounds which share at least two common epitopic sites, or a receptor.

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

Receptor—any compound or composition capable of recognizing a spatial and polar organization of a molecular i.e. epitopic site. In the subject invention, there will normally be two different receptors employed. The first receptor which will be labeled, will be monovalent, having only one binding site, which binding site is specific to a particular spatial and polar organization. For the most part, the monovalent receptors will be Fab fragments of antibodies, conveniently obtained by peptidase digestion of an antibody. Since for the most part, the monovalent receptors will be Fab fragments, these receptors will normally be referred to as Fab fragments, it being understood that Fab fragments is illustrative of a broader class of monovalent receptors. The other receptors which will be employed will normally be polyvalent receptors. These receptors include antibodies, enzymes, lectins, and the like. The receptor and its reciprocal ligand form a specific or homologous binding pair. In referring to receptors to a ligand, the receptors will be referred to as "antiligand."

Label—a molecule under about 5,000 molecular weight which is capable in combination with electromagnetic radiation or auxiliary chemical reagents of producing a detectible signal, which capability is affected by the presence of labeled or unlabeled receptor bound to ligand in spatial proximity to the label. Illustrative labels include fluorescers, chemiluminescers, nonenzymatic catalysts, groups having enzymatically labile bonds, and the like.

Label-Fab—a conjugate in which the label is covalently bound to a Fab antiligand, there being on the average at least one label per Fab antiligand.

Ligand-label complex—a complex having at least one label-Fab non-covalently bound to ligand and retaining at least one free epitopic site.

Ligand-Label Complex

The ligand-label complex has three components: (1) ligand; (2) monovalent receptor (abbreviated as Fab); and (3) label. The first component to be discussed will be the ligand.

Ligand

The ligands which will be employed in the subject invention will generally have at least 15,000 molecular weight, more usually at least 25,000 molecular weight and for the most part at least 50,000 molecular weight. There is no real upper limit on molecular weight, although most compositions which will be of diagnostic interest will generally be below 2,000,000 molecular weight, more usually below 1,000,000 molecular weight. The polyepitopic ligand analytes will normally be poly(amino acids) i.e. polypeptides and proteins, polysaccharides, nucleic acids, and combinations thereof. Such combinations or assemblages include bacteria, viruses, chromosomes, genes, mitchondria, nuclei, cell membranes, and the like.

For the most part, hormones of interest will generally be from about 20,000 to 100,000 molecular weight, more usually from about 20,000 to 60,000 molecular weight. Enzymes of interest will generally range from about 20,000 to 600,000 molecular weight, more usually from 20,000 to about 300,000 molecular weight. Immunoglobulins will generally range from about 150,000 to 1,000,000 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
- 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
- α_1X-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)
- 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)
Mol. formula:
($\delta_2\kappa_2$) or ($\delta_2\lambda_2$)
Immunoglobulin E (IgE) or γE-Globulin (γE)
Mol. formula:
($\epsilon_2\kappa_2$) or ($\epsilon_2\lambda_2$)
Free K and γ light chains
Complement factors:
C'1
C'1q
C'1r
C'1s
C'2
C'3
β_1A
α_2D
C'4
C'5
C'6
C'7
C'8
C'9
Important blood clotting factors include:

BLOOD CLOTTING FACTORS

International designation	Name
I	Fibrinogen
II	Prothrombin
IIa	Thrombin
III	Tissue thromboplastin
V and VI	Proaccelerin, accelerator globulin
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
Insulin
Glucagon
Relaxin
Erythropoietin
Melanotropin (melanocyte-stimulating hormone; intermedin)
Somatotropin (growth hormone)
Corticotropin (adrenocorticotropic hormone)
Thyrotropin
Follicle-stimulating hormone
Luteinizing hormone (interstitial cell-stimulating hormone)
Luteomammotropic hormone (luteotropin, prolactin)
Gonadotropin (chorionic gonadotropin)

Tissue Hormones

Secretin
Gastrin
Angiotensin I and II
Bradykinin
Human placental lactogen

Peptide Hormones from the Neurohypophysis

Oxytocin
Vasopressin
Releasing factors (RF), CRF, LRF, TRF, Somatotropin-RF, GRF, FSH-RF, PIF, MIF

Other polymeric materials of interest are mucopolysaccharides and polysaccharides.

Illustrative antigenic polysaccharides derived from microorganisms are as follows:

Species of Microorganisms	Hemosensitin Found in
Streptococcus pyogenes	Polysaccharide
Diplococcus pneumoniae	Polysaccharide
Neisseria meningitidis	Polysaccharide
Neisseria gonorrhoeae	Polysaccharide
Corynebacterium diphtheriae	Polysaccharide
Actinobacillus mallei; Actinobacillus whitemori	Crude extract
Francisella tularensis	Lipopolysaccharide Polysaccharide
Pasteurella pestis	
Pasteurella pestis	Polysaccharide
Pasteurella multocida	Capsular antigen
Brucella abortus	Crude extract
Haemophilus influenzae	Polysaccharide
Haemophilus pertussis	Crude
Treponema reiteri	Polysaccharide
Veillonella	Lipopolysaccharide
Erysipelothrix	Polysaccharide
Listeria monocytogenes	Polysaccharide
Chromobacterium	Lipopolysaccharide
Mycobacterium tuberculosis	Saline extract of 90% phenol extracted mycobacteria and polysaccharide fraction of cells and tuberculin
Klebsiella aerogenes	Polysaccharide
Klebsiella cloacae	Polysaccharide
Salmonella typhosa	Lipopolysaccharide, Polysaccharide
Salmonella typhi-murium; Salmonella derby Salmonella pullorum	Polysaccharide
Shigella dysenteriae	Polysaccharide
Shigella flexneri	
Shigella sonnei	Crude, polysaccharide
Rickettsiae	Crude extract
Candida albicans	Polysaccharide
Entamoeba histolytica	Crude extract

The microorganisms which are assayed may be intact, lysed, ground or otherwise fragmented, and the resulting composition or portion, e.g. by extraction, assayed. Microorganisms of interest include:

Corynebacteria

Corynebacterium diptheriae

Pneumococci

Diplococcus pneumoniae

Streptococci

Streptococcus pyogenes
Streptococcus salivarus

Staphylococci

Staphylococcus aureus
Staphylococcus albus

Neisseriae

Neisseria meningitidis
Neisseria gonorrheae

Enterobacteriaciae		
Escherichia coli	}	The coliform bacteria
Aerobacter aerogenes		
Klebsiella pneumoniae		
Salmonella typhosa	}	The Salmonellae
Salmonella choleraesuis		
Salmonella typhimurium		
Shigella dysenteriae	}	The Shigellae
Shigella schmitzii		
Shigella arabinotarda		
Shigella flexneri		
Shigella boydii		
Shigella Sonnei		
Other enteric bacilli		
Proteus vulgaris	}	Proteus species
Proteus mirabilis		
Proteus morgani		
Pseudomonas aeruginosa		
Alcaligenes faecalis		
Vibrio cholerae		

Hemophilus-Bordetella group

Hemophilus influenzae,
H. ducreyi
H. hemophilus
H. aegypticus
H. paraiufluenzae
Bordetella pertussis

Pasteurellae

Pasteurella pestis
Pasteurella tularensis

Brucellae

Brucella melitensis
Brucella abortus
Brucella suis

Aerobic Spore-forming Bacilli

Bacillus anthracis
Bacillus subtilis
Bacillus megaterium
Bacillus cereus

Anaerobic Spore-forming Bacilli

Clostridium botulinum
Clostridium tetani
Clostridium perfringens
Clostridium novyi
Clostridium septicum
Clostridium histolyticum
Clostridium tertium
Clostridium bifermentans
Clostridium sporogenes

Mycobacteria

Mycobacterium tuberculosis hominis
Mycobacterium bovis
Mycobacterium avium
Mycobacterium leprae
Mycobacterium paratuberculosis

Actinomycetes (fungus-like bacteria)

Actinomyces israelii
Actinomyces bovis
Actinomyces naeslundii
Nocardia asteroides
Nocardia brasiliensis

The Spirochetes

Treponema pallidum
Treponema pertenue
Treponema carateum
Borrelia recurrentis
Leptospira icterohemorrhagiae
Leptospira canicola
Spirillum minus
Streptobacillus moniliformis

Mycoplasmas

Mycoplasma pneumoniae

Other pathogens

Listeria monocytogenes
Erysipelothrix rhusiopathiae
Streptobacillus moniliformis
Donvania granulomatis
Bartonella bacilliformis

Rickettsiae (bacteria-like parasites)

Rickettsia prowazekii
Rickettsia mooseri
Rickettsia rickettsii
Rickettsia conori
Rickettsia australis
Rickettsia sibiricus
Rickettsia akari
Rickettsia tsutsugamushi
Rickettsia burnetii
Rickettsia quintana

Chlamydia (unclassifiable parasites bacterial/viral)

Chlamydia agents (naming uncertain)

Fungi		
Cryptococcus neoformans		
Blastomyces dermatidis		
Histoplasma capsulatum		
Coccidioides immitis		
Paracoccidiodes brasiliensis		
Candida albicans		
Aspergillus fumigatus		
Mucor corymbifer (Absidia corymbifera)		
Rhizopus oryzae	}	Phycomycetes
Rhizopus arrhizus		
Rhizopus nigricans		
Sporotrichum schenkii		
Fonsecaea pedrosoi		
Fonsecaea compacta		
Fonsecaea dermatitidis		
Cladosporium carrionii		
Phialophora verrucosa		
Aspergillus nidulans		
Madurella mycetomi		
Madurella grisea		
Allescheria boydii		
Phialosphora jeanselmei		
Microsporum gypseum		
Trichophyton mentagrophytes		
Keratinomyces ajelloi		
Microsporum canis		
Trichophyton rubrum		
Microsporum andouini		

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