Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 496-503

NEW APPROACH TO HETEROGENEOUS ENZYME IMMUNOASSAYS USING TAGGED ENZYME-LIGAND CONJUGATES

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Received February 12,1981

SUMMARY

A new approach to heterogeneous enzyme immunoassays has been developed that uses a tag molecule linked to an enzyme-ligand conjugate. The insoluble phase is an insolubilized receptor to that tag. The antibody to the ligand, in addition to complexing either the free ligand or the one covalently linked to the tagged enzyme, also serves to mask the tag on the tagged enzyme-ligand conjugate so that it can no longer bind to the insolubilized receptor. Accordingly, the proportion of enzyme conjugate associated with the insoluble fraction is proportional to the amount of analyte ligand being assayed. This heterogeneous EIA based on the "antibody masking the tag" is called AMETIA. In the model system we use DNP-lysine as the ligand, β-galactosidase as the enzyme, biotin as the tag, and avidin immobilized to Sepharose as the insoluble receptor.

Enzyme immunoassays (EIA) are usually categorized as either separation-free (homogeneous), i.e. not entailing a separation of the unbound ligand from that bound to the antibody, or separation (heterogeneous), i.e. those requiring such a separation process (1-11).

In heterogeneous EIA's described (1-4), the separation process depends on the ligand (antigen)-antibody reactions. For most EIA's these reactions take place at solid-liquid interphase because either the ligand (antigen) or the primary or secondary antibody is immobilized on a solid material. The enzyme in these heterogeneous EIA's is linked to ligand, antigen or antibody.

In this report we described a new approach to heterogeneous EIA, one that uses an enzyme label covalently linked not only to ligands, but also to "tag" molecules which can bind tightly to an insolubilized receptor.

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0006-291X/81/060496-08$01.00/0

MATERIALS AND METHODS

Chemicals and their sources were: 2,4-Dinitrophenyl-ε-lysine, o-nitrophenyl-β-D-galactopyranoside and avidin (Sigma Chemical Co.); E. coli β-galactosidase (Boehringer Mannheim Biochemicals); AH-Sepharose (Pharmacia Fine Chemicals); rabbbit anti-DNP serum (Miles Labs, Inc.); and m-Maleimidobenzoyl-N-hydroxy-succinimide ester (Pierce Chemical Co.).

Immobilization of avidin on aminohexyl-Sepharose (AH-Sepharose). Avidin was immobilized on AH-Sepharose using glutaraldehyde as a crosslinker. Washed AH-Sepharose (10 ml) was suspended in 400 ml 5% glutaradehyde in 0.2 M $NaHCO_3$, pH 8.5. The suspension was stirred for 1 h at room temperature (RT), after which the gel was washed successively with 0.5 ℓ 0.5 M NaCl and 0.5 ℓ 0.1 M $NaHCO_3$ pH 8.5. The glutaraldehyde activated gel was suspended in 5 ml avidin (50 mg in 5 ml 0.1 M $NaHCO_3$, pH 8.5), the suspension was stirred at RT for 1 h and at 4°C for 20 h. The avidin gel conjugate was washed sequentially with 1 ℓ 0.5 M NaCl in 0.1 M sodium phosphate, pH 8.0 and 1 ℓ 0.1 M sodium phosphate, pH 8.0. The amount of avidin bound was 13 mg/10 ml packed gel.

Synthesis of m-maleimidobenzoyl-DNP-lysine. Ten μmoles M-maleimidobenzoyl-N-hydroxysuccinimide ester, 11 μmoles Na_2CO_3 were added to 2 ml mixture of tetrahydrofuran and dimethylformamide (1:1 mixture) and stirred at RT. After 24 h the reaction was completed.

Labeling of β-galactosidase with biotin. β-Galactosidase was dialyzed against 0.1 M sodium phosphate, pH 8.0. To the dialyzed enzyme (2.8 nmoles in 2 ml 0.1 M phosphate, pH 8.0) was added 281 nmoles N-hydroxysuccinimide-biotin in 0.1 ml dimethylsulfoxide. The solution was stirred at RT for 3 h and at 4°C for 5 h, and then dialyzed at 4°C.

Labeling of biotinyl-β-galactosidase with m-maleimidobenzoyl-DNP-lysine. To 1 ml biotinyl enzyme (1.5 nmoles) was added 0.2 ml m-maleimidobenzoyl-DNP-lysine (1 μmole). The solution was stirred at RT for 2 h. The excess unreacted m-maleimidobenzoyl-DNP-lysine was separated from the labeled enzyme on a column (2 x 45 cm) of Sephadex G-50 C. The eluant was 0.1 M sodium phosphate, pH 7.2.

Enzyme activity. The enzyme activity in the supernatant or the insoluble fractions was assayed using o-nitrophenyl-β-galactopyranoside as a substrate (12).

RESULTS

The procedure outlined in Figure 1 is for preparing β-galactosidase (E) linked to biotin (tag) and to DNP-lysine (ligand). The enzyme (I), which possesses both free amino and thiol groups, was reacted with activated biotin (B), i.e. N-hydroxysuccinimidobiotin (II). The resultant biotinyl enzyme (III) was reacted with m-maleimidobenzoyl-DNP-lysine (VI) obtained by reacting m-maleidobenzoyl-N-hydroxysuccinimide ester (IV) with DNP-lysine (V). The final product was the tagged enzyme-ligand conjugate (VII) consisting of β-galactosidase linked to DNP-lysine (IV) through m-maleimidobenzoate and to biotin tags. Each molecule of enzyme was linked to 21 biotin tags and 37 DNP-lysines.

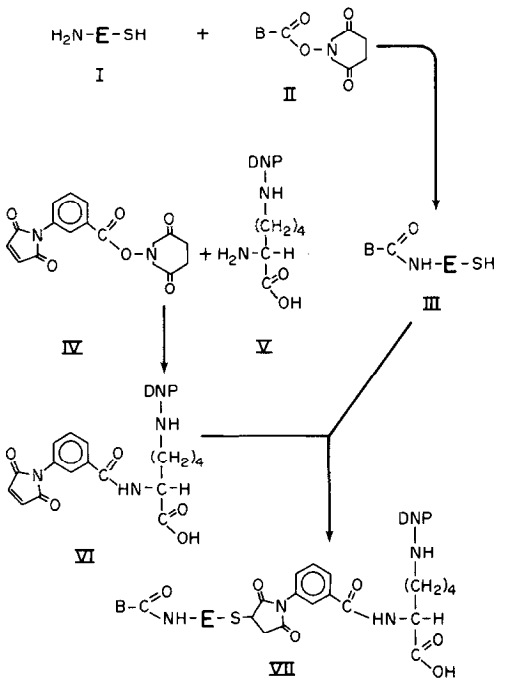


FIGURE 1: Chemical processes for preparing β-galactosidase linked to both DNP-lysine and biotin. I = β-galactosidase; II = N-hydroxysuccinimido-biotin; III = biotinyl-β-galactosidase; IV = m-maleimidobenzoyl-N-hydroxysuccinimide ester; V = DNP-lysine; VI = m-maleimidobenzoyl-DNP-lysine; VII = β-galactosidase labeled with biotin and DNP-lysine.

Addition of increasing amounts of avidin-gel, i.e. the insolubilized receptor, to a fixed concentration of biotin tagged enzyme-ligand conjugate gave a decrease in enzyme activity in the supernatant fraction and a concommitant increase in activity in the insoluble avidin-gel fraction (Fig. 2).

When fixed amounts of avidin-gel were added to solution containing fixed amounts of tagged enzyme-ligand conjugate and increasing amounts of anti-DNP serum, the enzyme activity in the supernatant increased and that of the insoluble fraction decreased (Fig. 3). Hence, these results demonstrate that (a) antibodies bound to the DNP-residue of the tagged enzyme-ligand conjugate, and (b) the biotin tag molecules of the resultant antibody conjugate complex was not able to bind to the receptor, avidin-gel, presumably because the antibody masked the biotinyl residues on the enzyme.

The DNP-lysine residues of the conjugate competed successfully for the antibodies with free analyte DNP-lysine. When increasing amounts of DNP-lysine

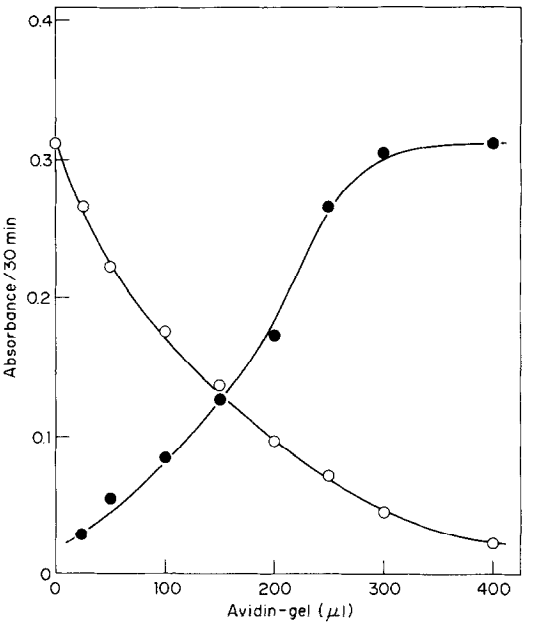
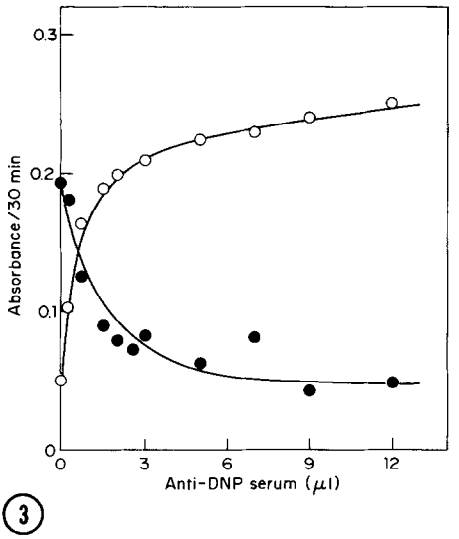


FIGURE 2: Binding of β-galactosidase linked to DNP-lysine and biotin to sepharose bound avidin. Varying amounts of 10% avidin-gel suspension were added to 200 μl β-galactosidase linked to DNP-lysine and biotin (1.36 nM). The suspensions were adjusted to 600 μl with 0.5% gelatin in 0.1 M sodium phosphate, pH 7.2 and incubated at 25°C for 30 min. The suspensions were centrifuged for 5 min using an Eppendorf centrifuge. The supernatants (500 μl) were assayed for β-galactosidase activity using o-nitrophenyl-β-galactopyranoside as the substrate (open circles). The pellets after centrifugation were washed by suspending them in 1 ml 0.5% gelatin in 0.1 M sodium phosphate, pH 7.2 followed by centrifugation for 5 5 min. The washing was repeated three times. The final pellets were assayed for enzyme activity by suspending them in 3 ml substrate solution (closed circles). All assays were incubated at 25°C for 30 min.

were added to fixed amounts of conjugate, antibody and avidin-gel, the enzyme activity in the supernatant decreased while simultaneously that in the insoluble fraction increased (Figure 4). Presumably by having DNP-lysine compete with ligands of tagged enzyme-ligand conjugates for antibodies, there are more conjugate uncomplexed with antibody and free to bind to insolubilized receptor. Thus, using the curve presented in Figure 4, the concentration of analyte, DNP-lysine in the range of 1-25 nM can be measured.

DISCUSSION

A new approach to the heterogeneous EIA's is described in which the insoluble fraction of the tagged enzyme-ligand conjugate is not derived from antigen-



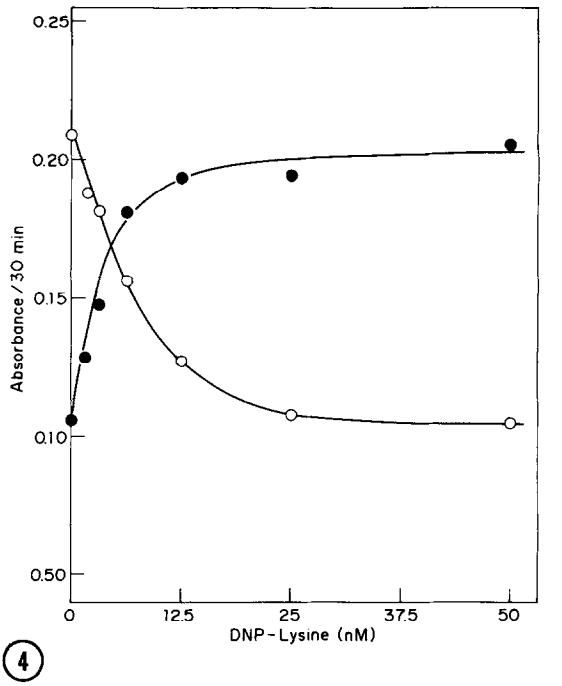


FIGURE 3: Antibody to DNP-lysine prevents the β-galactosidase linked to DNP-lysine and biotin from binding to avidin-gel. Varying amounts of anti-DNP serum were added to 200 μℓ β-galactosidase linked to DNP-lysine and biotin (1.36 nM). The solutions were adjusted to 600 μℓ. Constant amounts of 10% avidin-gel suspension (400 λ) were added. The mixtures were incubated at 25°C for 30 min and were centrifuged for 5 min. The supernatants (800 μℓ) were assayed for enzyme activities (open circles). The pellets were washed three times and assayed for activities (closed circles) as described in legend of Fig. 2.

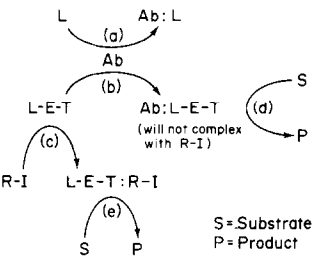
FIGURE 4: Standard curve for measuring DNP-lysine by AMETIA. Solutions of 200 μℓ containing various amounts of DNP-lysine were added to 200 μℓ solutions containing a fixed concentration of β-galactosidase linked to DNP-lysine and biotin (1.36 nM). To these solutions were added 0.5 μℓ anti-DNP serum and 500 μℓ 10% avidin-gel suspension. The mixtures were incubated at 25°C for 30 min and centrifuged for 5 min. The supernatants (800 μℓ) were assayed for enzyme activity (open circles). The pellets were washed three times and assayed for enzyme activity (closed circles) as described in legend of Fig. 2.

antibody interactions. Instead, the insoluble fraction results from the high affinity binding of a tag molecule, which is part of an enzyme-ligand conjugate, to an insolubilized receptor. In this assay the antibody for the ligand functions uniquely in preventing the tag of the conjugate from binding to the insolubilized receptor. Thus We call this assay "antibody masking enzyme tag immunoassay" (AMETIA).

Principles of AMETIA:

(i) Competition in a homogeneous solution phase for antibody (Ab) by ligand (L) and ligand of tagged enzyme-ligand conjugate (L-E-T), as in reactions (a) and (b) of scheme I.

SCHEME I



(ii) Separation by a brief centrifugation of antibody complexed tagged enzyme-ligand conjugate (Ab:L-E-T) from uncomplexed L-E-T by binding L-E-T to insolubilized receptor (R-I) to form L-E-T:R-I (reaction c). The binding between L-E-T and R-I occurs at a heterogeneous solid-liquid interphase. The T of Ab:L-E-T is not able to complex with receptor of R-I because of the presence of Ab.

(iii) Measuring the enzyme activity in either Ab:L-E-T of supernatant (reaction d), or L-E-T:R-I in the insoluble fraction (reaction e).

In AMETIA, a fixed concentration of L-E-T, Ab and R-I is used. The amount of free L-E-T available to complex R-I is dependent on the amount of L, because both L and L-E-T compete for Ab (reactions a and b). Thus, the lower the concentration of L, the more Ab are available to complex L-E-T (reaction b) so that less L-E-T is available to complex R-I. When L-E-T is complexed with Ab to form Ab:L-E-T by reaction b, it can no longer bind R-I, presumably because the Ab in Ab:L-E-T hinders physically the interaction between the tags and R-I.

Thus, it is obvious from Scheme I that a low concentration of L results in a high activity in the supernatant (reaction d) and low activity in the insoluble fraction (reaction e). On the other hand, more L will tie up more Ab (reaction a) freeing L-E-T so that it will complex with R-I (reaction c) resulting in higher activity in the insoluble fraction and less in the supernatant.

The results presented in Figures 2-4 bear out the validity of this approach to heterogeneous enzyme immunoassays.

Novelty of AMETIA:

All heterogeneous enzyme labeled immunoassays thus far described (1-4) utilize antigen or ligand-antibody bindings together with either immobilized antigen or ligand or immobilized antibody as a means of separating the unbound from the antibody bound fractions. In AMETIA, however, no ligand or antigen-antibody reaction was used in the separation step.

In AMETIA, the antibody serves two unique functions as regard to EIA. It serves to bind the analyte or an analyte derivative (L) of L-E-T as well as to mask the tag molecules (T) of L-E-T. The unique aspect of the separation step in AMETIA is the binding of the enzyme conjugate (L-E-T) that is not complexed with antibody to the insolubilized receptor (R-I) to form the insoluble L-E-T:R-I. The portion of L-E-T complexed with antibody (i.e. Ab:L-E-T) cannot bind to R-I, and hence, remains in the supernatant.

In all heterogeneous EIA's described (1-4, 13, 14, 16-19), the enzyme-ligand conjugates bound to the insoluble fractions are inevitably those complexed with antibodies. In AMETIA the opposite is true, i.e. the conjugates bound to the insoluble fraction are always those uncomplexed, free conjugates.

Another feature distinguishing AMETIA from other competitive heterogeneous EIA's is that at low analyte concentration the percentage of enzyme-ligand conjugate bound to the insoluble phase increases with increasing concentrations of the analyte. Therefore the standard curve in AMETIA is a line with positive slopes (Fig. 4, closed circles rather than one with negative slopes as is the case for other heterogeneous EIA (13, 14).

Molecules other than biotin can be used as tags provided high affinity receptors for them exist. Biotin-avidin system is ideal because of their extraordinary affinity to each other (15). One can tag the enzyme with avidin rather than with biotin and use immobilized biotin as a molecular hook to fish out the avidin tagged enzyme-ligand conjugate (L-E-T).

In AMETIA, the insolubilized receptor is a "universal" separating reagent because it can be used to develop EIA's for different ligands, as long as the enzyme is linked to the same tag. The centrifugation step in AMETIA can be eliminated by using a receptor coated tube or using a column of insolubilized receptor.

In conclusion, we have described a new approach using a tagged enzyme-ligand conjugate and an insolubilized receptor for the development of a sensitive and convenient heterogeneous EIA that provides a subtle and practical alternative to the techniques first developed by van Weemen and Schuurs (16-18) and by Engvall and Pearlmann (19).

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