MASTER

Titration of Tryptophan Oxygenase
Antigen by Immunoinhibition*

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SUMMARY

A method for determining tryptophan oxygenase as an antigen by the inhibition of its catalytic activity with an antibody is described. Unlike immunoprecipitation methods, this method does not require a pure antigen to obtain a single antibody against that antigen. Thus, it provides a means of measuring relative amounts of antigen in an enzyme preparation when the enzyme has not been purified to homogeneity. This titration method can also discriminate between the apo and holo forms of tryptophan oxygenase.

Immunochemical titrations of rat liver tryptophan oxygenase (EC 1.13.1.12) antigen have been reported by Feigelson and Greengard (1) and Schimke et al. (2). In their methods varying amounts of enzyme activity were added to a constant amount of antiserum. When the amount of antigen exceeded antibody, the antigen-antibody complex was removed by centrifugation and the appearance of the catalytic activity, measured in the supernatant, marked the endpoint of the titration. Knox and coworkers (3, 4) described an immunoinhibition titration of tryptophan oxygenase. It requires an antibody which inhibits the enzymatic activity but does not necessarily precipitate the enzyme protein. In this procedure small increments of antiserum were added to constant amounts of enzyme. This allowed the determination of low concentrations of antigen because the inhibition of catalytic activity by small amounts of antiserum could be easily detected. This paper develops the titration based on those principles. The variable factors have been investigated and analyzed. Appropriate controls have been incorporated into the procedure to correct for the major variable, the different affinities of apoenzyme and holoenzyme for the antibody.

MATERIALS AND METHODS

Inbred NEDH adult male rats, 200-250 g, were used. The crude preparation of tryptophan oxygenase was the soluble fraction of liver obtained by homogenizing liver in 3 volumes of 0.14 M KCl-0.02 M potassium phosphate buffer at pH 7.0, which was then centrifuged at 40,000 rpm for 1 hour in the Spinco Model, rotor 50. In some experiments the concentration of enzyme was increased by using livers of rats induced with hydrocortisone

or L-tryptophan (5). The total catalytic activity and activity due to the holoenzyme of tryptophan oxygenase were measured by the method of Piras and Knox (5). The partial purification of tryptophan oxygenase and its use for preparation of specific antibody against tryptophan oxygenase in rabbits have been described by Knox et al. (4). The antiserum was stable for months at -20° .

RESULTS

The tryptophan oxygenase antigen was determined by titration against a standardized antiserum which inhibited tryptophan oxygenase activity. The antigen titration, like the tryptophan oxygenase assay included in it, consists of two steps. The first is a preincubation to form the antigen-antibody complex and to activate that portion of enzyme which is not inhibited by the antibody. The second step is the assay of the tryptophan oxygenase catalytic activity remaining in the preparation. The reaction mixture was similar to that used for the conversion of tryptophan oxygenase to the catalytically active form (5): 0.5 mg methemoglobin, 5 mM L-tryptophan, 30 mM ascorbate, 10 mM reduced glutathione, tryptophan oxygenase antiserum, and 0.5 ml of 25% soluble fraction of liver (containing the enzyme) in a final volume of 1.0 ml. This mixture was incubated for 40 min at 37° . At the end of this preincubation, a 0.4-0.8 ml aliquot was added to a 10 mm cuvette containing 0.2 ml of 0.05 M L-tryptophan, 0.7 ml of 0.2 M phosphate buffer, pH 7, 0.1 ml of 0.3 M ascorbate and enough water for a total volume of 3.0 ml. The rate of increase in absorbancy at 360 nm due to kynurenine formation was followed for 10 min at 25° . A blank correction of 0.01 absorbancy units per 10 min was subtracted and the catalytic activity was expressed in units of μ moles kynurenine formed per hour at 25° .

Fig. 1. gives the reason for choosing a 40 min incubation period. When tryptophan oxygenase in the soluble fraction of liver was preincubated alone or with 0.04 ml of antiserum maximal activity was attained between 30 and 40 min. The activity was partially inhibited in the presence of the antiserum. Incubations longer than 40 min did not produce more activation or more inhibition of tryptophan oxygenase activity. It appeared that the formation of the antigen-antibody complex was essentially complete in the same period required for activation. The same result was obtained with conjugated enzyme, which appeared not to react more slowly with the antibody.

In Fig. 2, two concentrations of tryptophan oxygenase (from ^{liver}/soluble fractions of hydrocortisone-induced and noninduced rats) have been activated in the presence of increasing amounts of antiserum. The final enzyme catalytic activities in units per ml of preincubation solution have been plotted against the volume of antiserum added. The inhibition of tryptophan oxygenase activity by its antibody appears to be first order up to 80% inhibition. The insert shows the proportionality between the logarithm of the activity and volume of antiserum added.

One antibody unit is defined as the amount of antiserum necessary to inhibit by 50% one unit of tryptophan oxygenase catalytic activity in the standard enzyme preparation. This was a freshly prepared 25% soluble fraction of liver from a rat treated 5 hours earlier with 2.5 mg hydrocortisone phosphate per 100 g body weight. The enzyme therein is largely present as the apoenzyme (5). One antibody unit equals one unit of tryptophan oxygenase antigen, which in turn is equivalent to one unit of activity (1 μ mole/hour at 25°) in the standard enzyme preparation. Fig. 3 is a compilation of several titrations in which different antisera and different enzyme preparations were used. The enzyme activities were normalized

by converting each activity measurement in the presence of antibody to a percent of its activity without antibody. Units of antibody were expressed as multiples of the amount of antiserum required to inhibit one activity unit 50%. The inhibition of tryptophan oxygenase activity was first order from 0-80%.

The endpoint of a tryptophan oxygenase antigen titration is the volume of antiserum for 50% inhibition of the total activity, Ab_{50} . This value can be determined from a semilogarithmic plot of activity versus volume of antiserum added, as in Figs. 2 or 3, or it can be calculated, with greater objectivity, by a linear regression of the logarithms of activity on antiserum volumes. From the equation of the straight line, the Y-intercept is the averaged logarithm of tryptophan oxygenase activity without antiserum; and the endpoint of the titration, Ab_{50} , is $\log 2$ divided by the absolute value of the slope. The values obtained by these two methods were comparable. Fig. 4 shows that antibody units are proportional to activity units from 0.5-5.0 for fresh and almost equally so for frozen standard enzyme preparations. Standard fresh preparations of tryptophan oxygenase were titrated a number of times in this way to obtain the mean antibody titer for a given preparation of antiserum.

Standardized antisera were used to determine the amount of tryptophan oxygenase antigen in other types of enzyme preparations. By definition the ^{liver} soluble/fraction of hydrocortisone-induced rats contains the same amount of enzyme by both the catalytic and immunoinhibition assays and has an antigen to activity ratio (TO_{AG}/TO) of 1.0. Table I compares the contents and ratios of antigen and activity in a number of different enzyme preparations. The assay was not affected and the ratio of TO_{AG}/TO was not

different from 1.0 when hydrocortisone-induced or non-induced liver preparations were titrated over a range of protein concentrations, but in other types of preparations ratios greater than 1.0 were found. The greatest elevation (2.30) occurred in tryptophan-induced preparations. These contain the enzyme in a highly conjugated form (5). Ratios greater than 1.0 also occurred in standard preparations that had been frozen and at all stages of purification of the enzyme. Particularly instructive was the effect of a prior incubation with DL- α -methyltryptophan. This analog of the substrate permits conjugation of the enzyme (5). Although a 2 hour incubation of the standard preparation alone caused proportionate losses of both antigen and activity, the presence of α -methyltryptophan during the incubation appeared preferentially to preserve the antigen and raised the TO_{AG}/TO ratio above 1.0.

The amount of conjugated enzyme present in these preparations was measured in the presence of globin, which was used to prevent addition of heme to the apoenzyme during the assay (5). Tryptophan oxygenase preparations which had antigen to activity ratios greater than 1 also had high proportions of holoenzyme. For example, the standard hydrocortisone-induced soluble fraction with the TO_{AG}/TO ratio defined as 1.0 was less than 10% holoenzyme; a tryptophan-induced fraction had a TO_{AG}/TO ratio of 2.3 and was 70% holoenzyme. Incubation with α -methyltryptophan increased both the conjugation and the ratio. Variable ratios found in rats after hydrocortisone plus tryptophan induction was traced to changing conjugation as the administered tryptophan was metabolized. When the amount of antiserum required to inhibit each unit of tryptophan oxygenase ($Ab_{50}/Total\ TO$) in a variety of preparations was plotted against the fraction of enzyme initially present

as holoenzyme, the significant relationship in Fig. 5 was obtained. The equation of the line determined by least squares was $Y = 14.6 + 7.62$. The Y-intercept of 7.62 μ l is the theoretical amount of antiserum per unit of completely unconjugated enzyme. This value is in reasonable agreement with the standardized Ab_{50} of the antiserum used (7.9 μ l). Extrapolation to the fully conjugated state indicated that about three times more antibody was required to inhibit the holoenzyme than the apoenzyme (22.2 compared to 7.6 μ l per unit TO), or an apparent increase of 1.92 antigen units when one unit of apoenzyme was converted to the holoenzyme ($(22.2 - 7.6)/7.6 = 1.92$). Expressed in antigen units this relation was independent of the particular titers of different antisera.

It was more accurate in practice to correct for the initial holoenzyme content of a preparation than to conjugate it fully before the titration. A variety of preparations with measured degrees of conjugation could then be used as in Fig. 5 to standardize an antiserum to one unit of the apoenzyme, which is similar but more precise than use of the standard enzyme preparation. The antigen units expressed as apoenzyme in a preparation were then related to the volumes of antiserum for 50% inhibition of the unknown (Ab_{50} observed) and of the apoenzyme standard (Ab_{50} standard):

$$\text{Antigen units} = \frac{Ab_{50} \text{ Observed}}{1.92 (\% \text{ holoenzyme}/100) + 1.00} \times \frac{1}{Ab_{50} \text{ Standard}}$$

Table II contains some antigen assays which have been corrected in this way for the fractions of holoenzyme in the preparations. Assays of the largely unconjugated enzyme of hydrocortisone-treated livers were not affected. The apparent antigen in tryptophan-treated and in purified preparations was diminished by the correction to apoenzyme and the antigen to activity ratios

became nearly 1.0. The anomalous behavior of a hydrocortisone-induced preparation when incubated before the assay (Table I) was clarified. Conjugation, which occurred during the incubation, caused relatively high apparent antigen contents. However, the corrected contents of antigen as apoenzyme, even with α -methyltryptophan present during the incubation, were approximately equal to the activity measured.

DISCUSSION

This immunochemical titration for tryptophan oxygenase antigen is based solely on the ability of the antibody to inhibit the catalytic activity of the enzyme. Since antibodies synthesized against contaminating proteins will presumably not inhibit the enzyme, there is less concern about non-specific interactions than in immunoprecipitation methods. The enzyme activity may be endogenous in the sample or it may be added to a test solution suspected of containing inactive enzyme. The titration can in this way detect antigenic protein which has no catalytic activity by its competition with the active form of the enzyme for antibody. The inactive antigen is the difference between the total antigen, determined by the titration, and the active enzyme, determined by activity measurements. Relative values, based on the activity of standard enzyme preparations, are used instead of absolute amounts of enzyme protein, because pure enzyme and antibody are not available for quantitation. This method may be useful for measuring the antigen content of other enzyme preparations which have not been purified to homogeneity.

Under some conditions, inhibition of activity may be more sensitive to changes in antigen content than other methods. For example, we were able to detect loss of tryptophan oxygenase antigen in cell-free liver preparations

after an incubation (Table II) while Schimke et al. (2) did not observe a loss of antigen under similar incubation conditions with the classical type of immunoprecipitation assay.

A modification of the simple procedure corrected the additional anti-serum needed to inhibit the catalytic activity of the holoenzyme as compared to the apoenzyme. Only one possible reason for this different reaction of the apo- and holoenzymes appeared to be excluded. The time course of antibody inactivation of holoenzyme indicated that its reaction with antibody, like that of the apoenzyme (Fig. 1), was complete in 30-40 min. Other possible explanations of the requirement of holoenzyme for more antibody include the existence of several types of antibodies with different affinities for the apo- and holoenzymes. Although no qualitative differences of this kind were found among several different antisera, this possibility cannot be excluded. It is equally possible that a change in protein configuration from apo- to holoenzyme results in less affinity of the same antibody for the active site of the enzyme. Not enough is known about the configuration of the enzyme to interpret, in this way, the three-fold increase in antibody required for inhibition. But these results suggest that all forms of an enzyme, particularly if it has cofactors, need not interact equally well with an antiserum. Errors due to such heterogeneities could occur in any titration method based on inactivation or precipitation except when pure precipitating antibody is added in excess to an antigen and the amount of precipitate determined in some specific manner.

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FOOTNOTES

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LEGENDS

FIG. 1. Activation of tryptophan oxygenase in the presence and absence of specific antiserum. Tryptophan oxygenase activities (T.O.), as percent of the maximum, have been plotted against the duration of the preincubation period. The activities of T.O. in the absence of antibody ($-O-$); in the presence of 0.04 ml of antiserum ($-X-$).

FIG. 2. Inhibition of tryptophan oxygenase activity by a specific antibody. Soluble fractions of 2 livers, hydrocortisone-induced (O) and noninduced (X), were activated in the presence of increasing amounts of antiserum. The tryptophan oxygenase (T.O.) activities (μ moles/hour in 1 ml preincubation solution) are plotted against the ml of antiserum added. The insert is a semilogarithmic plot of the same data.

FIG. 3. Titrations of tryptophan oxygenase activity. The percent uninhibited tryptophan oxygenase (T.O.) activity (100% is without antibody) is plotted against antibody units added per unit of initial enzyme activity. Each point represents one point in a titration. Three different antisera (Δ , $\square$, O) were used to titrate two T.O. preparations (shown by open and filled symbols). The range of uninhibited T.O. activities was 1.4-7.9 units per assay (ml of preincubation solution). The 50% inhibition point of the initial T.O. activity is defined as the endpoint of the titration (Ab_{50}), at which one antibody unit inhibits one T.O. unit by 50%.

FIG. 4. Relationship of tryptophan oxygenase activity units to antibody units. Three antibody (Ab) preparations (O, Δ , $\square$) were used to titrate fresh, hydrocortisone-induced tryptophan oxygenase (T.O.), and also frozen

and non-induced (0) preparations. The average amounts of the three antisera required to inhibit one unit of activity 50% were 0.0258, 0.0138 and 0.0085 ml, giving antibody units per ml antiserum of 38.7, 72.5 and 117, respectively.

FIG. 5. Amounts of antiserum required to inhibit tryptophan oxygenase as a function of the holoenzyme fraction initially present. Ab_{50} of the antiserum used was 7.9 μ l (required to inhibit each standard unit of activity by 50%). The 19 different tryptophan oxygenase preparations used were hydrocortisone, hydrocortisone + tryptophan or tryptophan-induced soluble fractions, with the conjugated fraction (Holo T.O./Total T.O.) determined for each. The correlation coefficient, $r = 0.91$ (d.f. = 17, $P < 0.001$), is highly significant. The best line was determined by least squares. The Y-intercept, Ab_{50}/Apo T.O. is 7.62 μ l; the slope is 14.6.

TABLE I
Comparisons of activity and antigen units in various
preparations of tryptophan oxygenase

Mean and standard deviations of 4-23 measurements of activity and antigen in the soluble fraction of each type of preparation, and the ratio of these measures, is tabled. The ratio is 1.0 by definition in the fresh hydrocortisone-induced livers taken as the standard. This assay contained 0.5 ml of a 25% extract with about 12.5 mg protein per ml. Purification steps are those described by Knox *et al.* (4).

Preparation	T.O. ^a Activity	T.O. Antigen	<u>Antigen</u> Activity
	(units/ml of assay $\pm$ SD)		
Soluble fraction of liver			
Hydrocortisone-induced	2.02 $\pm$ 0.80	2.03 $\pm$ 0.95	1.00
Same with 4-19 mg protein/ml	0.48 to 7.95	0.49 to 8.16	1.06
Non-induced	0.73 $\pm$ 0.33	0.75 $\pm$ 0.27	1.03
Tryptophan-induced ^b	2.04 $\pm$ 0.15	4.68 $\pm$ 0.70	2.30
Hydrocortisone-induced, frozen	3.26 $\pm$ 0.30	4.10 $\pm$ 0.68	1.26
Purified T.O.			
pH 5 step	0.40 to 3.05	0.65 to 7.68	1.96
pH 5 step ^c	2.50 to 6.60	2.96 to 10.24	1.23
Calcium phosphate step	1.65 to 2.10	2.62 to 4.27	1.82
DEAE step	1.65 to 1.90	2.93 to 3.90	1.86
Incubated soluble fraction ^d	(% Initial Value)		
0 hour	100	100	1.00
2 hour	42 $\pm$ 20	47 $\pm$ 17	1.12
2 hour + α -methyltryptophan	71 $\pm$ 18	106 $\pm$ 18	1.50

^a Tryptophan oxygenase

^b 5 hour tryptophan induction in adrenalectomized rats

^c Assayed in the presence of 0.5 ml 25% non-induced extract per ml assay

^d Incubated 25% hydrocortisone-induced soluble fraction at 37°, pH 7
before assay

TABLE II

Correction of assays of tryptophan oxygenase for holoenzyme content

Results in single preparations are given without correction (apparent antigen) and with correction to the apoenzyme form as described in the text. Activity units, $\mu\text{moles/hour per ml}$, are equal to the antigen units in the standard assay ($\text{TO}_{\text{AG}}/\text{TO} = 1.0$). Hydrocortisone-induced preparations were also incubated for 2 hours at 37° , with and without 5 mM DL- α -methyltryptophan (OMeTrp), before the assays.

Preparation	Activity (T.O. ^a)	% Holo-TO	Apparent antigen (TO_{AG})		Antigen as apoenzyme (TO_{AG})	
	units/ml		TO_{AG}	$\text{TO}_{\text{AG}}/\text{TO}$	TO_{AG}	$\text{TO}_{\text{AG}}/\text{TO}$
Tryptophan-induced	1.79	78	4.37	2.44	1.81	1.01
Tryptophan-induced	2.13	91	5.51	2.59	1.98	0.93
Hydrocortisone-induced						
0 hour incubation	2.11	4.7	2.20	1.04	2.09	0.99
2 hour incubation	0.69	44	1.39	2.01	0.79	1.14
2 hour + OMeTrp incubation	2.07	41	3.66	1.77	2.12	1.02
Purified TO (CaP step)	2.10	62	4.27	2.03	2.02	0.96

^a Tryptophan oxygenase