Three Immunologically Distinct Isozymes of Phenylalanine Hydroxylase†

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ABSTRACT: Three immunologically distinct and non-cross-reacting isozymes of rat liver phenylalanine hydroxylase (PH) can be distinguished by immunotitration, immunodiffusion, and immunoabsorption. Only a single isozyme can be distinguished for rat kidney and minimal deviation hepatoma H4-II-E-C3 cell culture PH, by immunodiffusion and immunotitration. The ratios of antigen to antibody in milliunits of activity per milligram of immunoglobulin near equivalence by immunodiffusion of liver isozymes are 63, 21, 2.0, and 1.9 for kidney. The kidney (1.9 mU/mg of immunoglobulin) and the second liver (21 mU/mg of immunoglobulin) immunoprecipitin lines share immunologic identity as evidenced by their complete fusion. Immunoabsorption of the immunoglobulin with kidney PH with a ratio of antigen to antibody determined from near equivalence values obtained from immunotitration and immunodiffusion results abolishes the kidney and the second liver (21 mU/mg of immunoglobulin) immunoprecipitin lines. Additionally, retitration of this immunoabsorbed immunoglobulin abolishes all of the inhibition of kidney and shifts the initial immunotitration slope of liver PH to the left, but does not alter the second (2 mU/mg of immunoglobulin) slope. Immunotitration of H4-II-E-C3 minimal deviation hepatoma PH results in one slope with a near equivalence ratio of 2.1 mU/mg of immunoglobulin. No immunoprecipitin lines were observed on immunodiffusion for any of the dilutions of antigen or antibody tested. The behavior of H4-II-E-C3 PH is similar to antigen 3 of liver, and does not cross-react with the two other antibodies of liver.

Phenylalanine hydroxylase (PH)[1] catalyzes the initial degradation of the essential amino acid phenylalanine to tyrosine. Phenylketonuria (PKU) and its variants are a heterogeneous group of genetically determined human and metabolic diseases where there is a spectrum of deficient hepatic PH activity. The PH activity in PKU varies from virtual absence (Jervis, 1953; Friedman et al., 1973) to 5–10% of normal activity (Justice et al., 1967; Kang et al., 1970). The distribution of PH in mammalian tissues is confined to the liver, kidney, and pancreas (Tourian et al., 1969), and in humans to liver and kidney (Ayling et al., 1974). Kaufman and Fisher (1970) have identified two isozymes of PH by acrylamide gel electrophoresis. Barranger et al. (1972) have identified three isozymes by chromatography. It was also possible to show two isozymes of human fetal liver. Treiman and Tourian (1972) identified three isozymes of mouse liver by acrylamide gel electrophoresis. These isozymes are said to have identical molecular weight and kinetic constants, but differ in charge (Kaufman and Fisher, 1970; Barranger et al., 1972). However, there has been no previous identification of immunologically distinct isozymes of PH (Friedman et al., 1972; Ayling et al., 1974).

We have purified rat liver PH by the method of Kaufman and Fisher (1970) and prepared antibody by the method of Friedman et al. (1972). The immune serum from sheep was purified and used for the immunological studies. A purification step of ammonium sulfate fractionation of rat liver, minimal deviation hepatoma cell culture H4-II-E-C3, and kidney, which contains all of the initial tissue extract PH activity (Gillam et al., 1974), was used for the immunological studies.

We report finding three immunologically distinct and non-cross-reacting isozymes of rat liver PH, one of which shares immunological identity with rat kidney PH on immunodiffusion. Additionally, because of a tenfold difference between the liver and kidney isozyme equivalence values, it is unlikely that the kidney isozyme is identical with the second liver isozyme with which it cross-reacts. The evidence is based on immunotitration, immunoabsorption, and double immunodiffusion of liver and kidney ammonium sulfate fraction PH with purified sheep immunoglobulin.

Materials and Methods

Rat liver PH was purified by the method of Kaufman and Fisher (1970). PH was assayed by a previously published method (Tourian et al., 1972; Tourian, 1973). The specific activity of the Sephadex G-200 column step was 1.3 U per mg of protein per min. A unit of activity is defined as 1 μmol of tyrosine formed/min in 0.1 *M* potassium phosphate (pH 7.2) and 25°. This specific activity compares favorably with Kaufman and Fisher's (1970), 0.59 U, using 2 m*M* of L-phenylalanine for substrate concentration, compared to 8 m*M* for the present method.

Ammonium sulfate fractionation of 5-week male rat liver and kidney PH was prepared by the method of Gillam et al. (1974). The specific activity of this fraction was 109 mU/mg for liver and 16.9 mU/mg for kidney. Minimal deviation hepatoma H4-II-E-C3 cell culture extract was obtained and ammonium sulfate fractionated by a method that will be described in detail (Tourian, 1975). The specific activity of this fraction was 17.4 mU/mg. The ammonium sulfate fractions contained all of the initial enzyme activity of the respective tissues.

Preparation of Antibody. Rat liver purified enzyme was

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[1] Abbreviations used are: PH, phenylalanine hydroxylase; PKU, phenylketonuria.

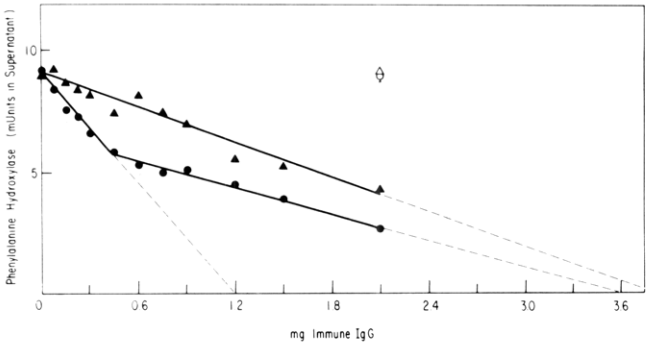

FIGURE 1: Immunotitration of liver and kidney PH. Liver or kidney PH (9 mU) was added to each incubation mixture in 150 μl final volume to increasing amounts of immunoglobulin. Control non-immune globulin was added to equalize the immunoglobulin of each incubation which also contained 0.1 *M* potassium phosphate (pH 6.8). After 4 hr at 0–4°, the precipitated antigen–antibody complex was centrifuged at 3500*g* for 20 min and 50 μl of the supernatant was assayed in duplicate for PH activity. Kidney PH (▲); liver PH (●); control non-immune globulin: kidney (△), liver (○).

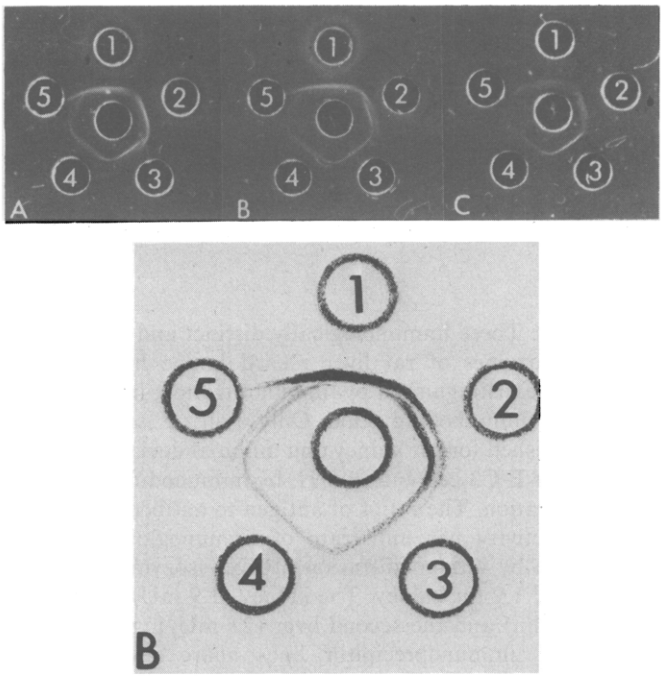

FIGURE 2: Double immunodiffusion of liver and kidney PH with immunoglobulin. The center well was filled with 150 μg of immunoglobulin and the peripheral wells (clockwise starting with well 1) with serial dilutions of (A) 1, 1: 1.5, 1:4.5, 1:9, 1:15; (B) 1:1.5, 1:4, 1:9, 1:17, 1:40 of 14.2 mU of liver PH; and (C) 1, 1:1.5, 1:4.5, 1:9, 1:15 of 1.3 mU of kidney PH. The immunodiffusion was allowed to proceed for 17 hr at 25°. Figure 2B has been drawn for clarity of antigen 3 in well 5. Control non-immune globulin (150 μg) was filled in the central wells, and identical serial dilutions of liver and kidney PH as in (A), (B), and (C) were added to the peripheral wells. No immunoprecipitin lines were observed at any of the antigen dilutions.

put on acrylamide gels and electrophoresed by previously published methods (Kaufman and Fisher, 1970; Friedman et al., 1972). The region of gel which had PH activity by direct measurement of enzymatic activity was cut from parallel gels, homogenized, and injected with Freund's adjuvant intramuscularly into a ram, after control serum had been obtained (Friedman et al., 1972). The rise in titer of antibody was measured by the microcomplement fixation of immune serum by a modification of the microtechnique of Sever (1962), which showed a positive titer at 60,000-fold dilution of serum after 4 weeks. Titers were determined by using a constant antigen concentration of 0.3 mg/ml in the presence of 8 units of complement against increasing twofold dilutions of whole serum previously treated by the method of Bucca and Adler (1969) to remove the anticomplementary activity. Under these conditions, each serum sample had to have a complement fixation titer of at least 8 before it would precipitate the antigen in immunodiffusion.

Immunodiffusion. Hyland plates of 2% Noble agar were used; 5 μl of PH or immunoglobulin at the indicated concentrations was put in each well. Immunodiffusion was allowed to proceed for 17 hr at 25°.

Purification of Immune Serum. The immune and control sera were fractionated by salting out with sodium sulfate and the antibody was purified as the immunoglobulin fraction (Kabat, 1961). The purification procedure yielded approximately 1 mg of immunoglobulin for every 100 μl of immune serum.

Preparation of Kidney PH Immunoabsorbed Immunoglobulin. Immunoglobulin (30 mg) was incubated with 76.8 mU of kidney PH ammonium sulfate fraction. The ratio of enzyme to immunoglobulin was obtained from the immunotitration curve of kidney PH from Figure 1, and incubated in 0.1 *M* phosphate buffer (pH 6.8) diluted to a final volume of 1.0 ml.

At the end of 48 hr, the precipitated antigen–antibody complex was centrifuged. The supernatants were brought to 33% ammonium sulfate and centrifuged, and the precipitated immunoglobulin was dissolved in 0.1 *M* potassium phosphate (pH 6.8). The concentration was adjusted to the previous immunoglobulin described above.

The kidney immunoabsorbed immunoglobulin was tested and found to be virtually free of any kidney PH by both activity (less than 3%) and by double immunodiffusion experiments. In the latter, the immunoabsorbed immunoglobulin was used at two concentrations of 15 and 30 μg/ml in a central well, and the immunoglobulin (not immunoabsorbed) in the peripheral well was serially diluted 16-fold. No residual kidney PH was present by this method, as evidenced by the lack of immunoprecipitin lines at any of the serial dilutions tested for each of the two concentrations of immunoabsorbed immunoglobulin.

Results

Immunotitration of Liver and Kidney PH with Immunoglobulin. Preliminary experiments testing the stability of the liver and kidney ammonium sulfate PH revealed the enzyme to be unstable with loss of activity when incubated at 0° for 17 hr or longer. However, it was stable when limited to a period of 4 hr. Thus all of the immunotitration incubations were limited to 4 hr. This period of incubation of the enzyme–antibody mixture resulted in sufficient immunoprecipitation of the antigen–antibody complex to make a quantitative evaluation possible.

A constant amount of 9 mU of PH was incubated with increasing amounts of immunoglobulin, keeping the concentration of immunoglobulin constant in each of the tubes by adding non-immune control globulin. The antigen–antibody complex was removed by centrifugation after 4 hr at 0° and the residual PH activity determined in the supernatant. Immunotitration of kidney PH results in a single slope, whereas the liver PH has two slopes (Figure 1). Similar results were obtained when tissue extract was used as the source of enzyme activity. No inhibition of PH activity was observed when control non-immune globulin was incubated at the

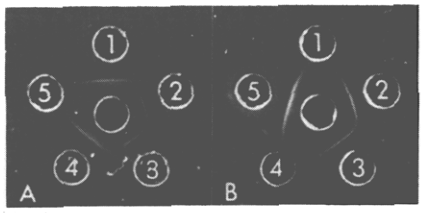

FIGURE 3: The identity of the inner immunoprecipitin line of liver with that of kidney PH on double immunodiffusion. The center well was filled with 150 μg of immunoglobulin. (A) Wells 1 and 4 were filled with 1:4.5 dilution of 14.2 mU of liver PH and wells 2, 3, and 5 were filled with 1.3 mU of kidney PH. The immunodiffusion was allowed to proceed for 17 hr at 25°. (B) Wells 1 and 4 were filled with 1:40, well 2 with 1:4 and well 5 with 1:1.5 dilutions of 14.2 mU of liver PH. Well 3 was filled with 1:4 dilution of 1.3 mU of kidney PH.

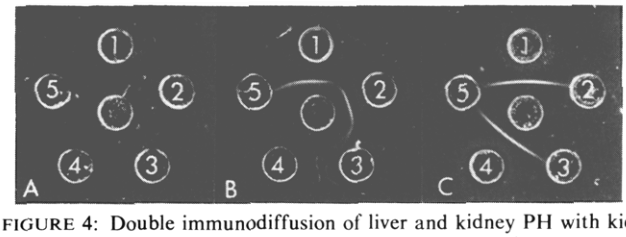

FIGURE 4: Double immunodiffusion of liver and kidney PH with kidney immunoabsorbed immunoglobulin. In Figure 4A and B the experimental conditions were the same as in Figure 2A and C, except that the center well was filled with 150 μg of kidney immunoabsorbed immunoglobulin as described under Materials and Methods. The peripheral wells of (A) were filled with 1, 1:1.5, 1:4.5, 1:9, 1:15 dilutions of 1.3 mU of kidney PH, and (B) with 1, 1:1.5, 1:4.5, 1:9, 1:15 dilutions of liver PH. Parallel plates were run for each condition and kept in the refrigerator at 4° after the initial 17 hr at room temperature for 3–4 weeks and periodically inspected. There was no change from the photograph displayed here which was taken at the end of 17 hr. (C) The experimental conditions were the same as in Figure 3A, except that the center well was filled with 150 μg of kidney immunoabsorbed immunoglobulin; wells 1 and 4 were filled with 1:4.5 dilution of 14.2 mU of liver PH and wells 2, 3, and 5 were filled with 1.3 mU of kidney PH. The plates were treated identically as in Figure 4A and B.

maximum concentration of immunoglobulin, or the antigen–antibody complex resuspended without centrifugation and its activity determined.

Immunodiffusion of Liver and Kidney PH. Double immunodiffusion of liver PH results in three lines, two strong and one weak (Figure 2). The first two are best seen in wells 2 and 3 of Figure 2A and at antigen dilutions of 1:1.5 and 1:4.5, respectively, and the third weak line is best seen in well 5 of Figure 2B and at an antigen dilution of 1:40. The ratios of enzyme activity to mg of immunoglobulin near equivalence for these immunoprecipitin lines are 63 and 21 mU/mg of immunoglobulin, respectively, for the first and second immunoprecipitin lines, and 2 mU/mg of immunoglobulin for the third. No immunoprecipitin lines were observed when control non-immune serum was added to the central well under identical conditions (not shown).

Double immunodiffusion of kidney PH results in one immunoprecipitin line (Figure 2C), even at higher and lower ratios of antigen to antibody than those shown. The ratio of enzyme activity to mg of immunoglobulin near equivalence for the immunoprecipitin line is 1.89 mU/mg of immunoglobulin in well 3 (Figure 2C).

The second immunoprecipitin line of liver PH completely fuses with that of the kidney line when appropriate dilutions of each of the enzymes are added to the immunodiffusion plates (Figure 3A and B); e.g., the fusion between wells 2 and 3 of Figure 3B in which liver and kidney extracts are compared.

Double Immunodiffusion of Liver and Kidney PH with Kidney PH Immunoabsorbed Immunoglobulin. The immunoabsorbed immunoglobulin solution was put in the central well and was tested for the presence of kidney and liver specific antibodies by double immunodiffusion, using a 15-fold serial dilution of liver or kidney PH in the peripheral wells. The conditions were identical as in the previous immunodiffusion experiment, where the immunoglobulin had not been immunoabsorbed (Figure 2A and C). There was no kidney immunoprecipitin line for any of the dilutions tested (Figure 4A). The first liver immunoprecipitin line was present; however, the second immunoprecipitin line was completely abolished (Figure 4B). The third liver immunoprecipitin line (Figure 4B, well 5) was not present; however, the same amount of specific antibody to liver antigen 1 was still present in the immunoabsorbed immunoglobulin, as evidenced by the presence of the second slope (2 mU/mg of immunoglobulin) in the immunotitration experiment shown in the following section. In another set of immunodiffusion plates, the central well was filled with 1.3 mU of kidney PH and tested with a 16-fold serial dilution of 75 or 150 μg of

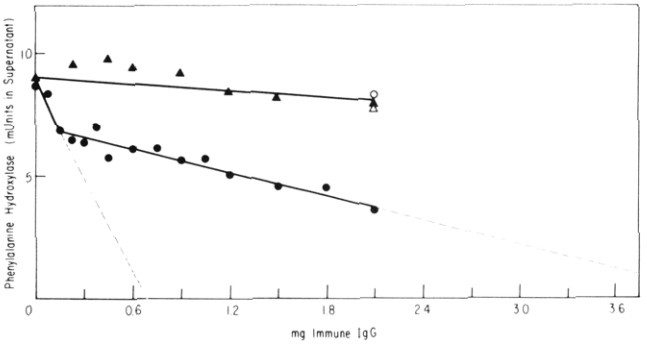

FIGURE 5: Immunotitration of liver and kidney PH with kidney immunoabsorbed immunoglobulin. The experimental conditions were the same as for Figure 1, except for the immunoabsorbed immunoglobulin which was prepared as described under Materials and Methods. Kidney PH (▲); liver PH (●); control non-immune globulin (Δ,0).

the immunoabsorbed immunoglobulin in the peripheral wells. No immunoprecipitin lines for kidney PH were observed (not shown).

Liver PH at the ratio of the second immunoprecipitin line (21 mU/mg of immunoglobulin) and kidney at 1.89 mU/mg were added to the peripheral wells and kidney immunoabsorbed immunoglobulin at the central well. The kidney and the second liver immunoprecipitin lines were completely abolished (Figure 4C; compare with Figure 3A).

Immunotitration of Liver and Kidney PH with Kidney Immunoabsorbed Immunoglobulin. Immunotitration of kidney and liver PH with kidney immunoabsorbed immunoglobulin demonstrates the complete absence of antibody to kidney PH and a shift to the left of the initial slope of liver immunotitration with no change in the second slope (Figure 5). Thus it is clear that the apparent biphasic curve of liver PH in Figure 1 is truly due to two separate liver enzymes; one (antigen 2) cross-reacts with kidney and the other (antigen 3) is specific. Since the liver PH curve remains biphasic in Figure 5, though shortened, it would appear that liver component 1 seen in immunodiffusion is also enzymatic and contributes to the steep drop in activity with 0.1 mg of absorbed immunoglobulin.

Thus, the preceding results define three enzymes participating in antigen–antibody interaction for the liver and one

Table I: Ratio of Phenylalanine Hydroxylase Activity to Milligrams of Immunoglobulin Near Equivalence from Immunodiffusion and the Extrapolated Value from Immunotitration Slopes.

Type of IgG[a]	Antigen Tested	Double Immunodiffusion	Immunotitration		
		Equivalence Ratio of mU of Enzyme Activity/mg of IgG	Equivalence Ratio of mU of Enzyme Act./mg of IgG	Observed Slopes	Predicted Slopes
Immune IgG	Kidney	1.9	2.4	One	One
Immune IgG	Liver	63.0 21.0 2.0	7.7 1.8	Two	Three
Kidney immuno-absorbed immune IgG	Kidney	None	None	None	None
Kidney immuno-absorbed immune IgG	Liver	60.0 None 2.0 (?)	13.2 None 1.8	Two	Two
Immune IgG	Minimal deviation hepatoma H4-II-E-C3		2.1	One	One

[a] The designation of IgG for immunoglobulin is for abbreviation purposes only.

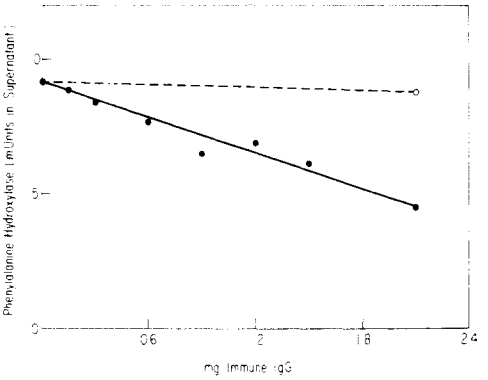

FIGURE 6: Immunotitration of H4-II-E-C3 minimal deviation hepatoma tissue culture PH. H4-II-E-C3 PH (9 mU) was added to each incubation mixture in 150 μl final volume to increasing amounts of immunoglobulin. Control non-immune globulin was added to equalize the immunoglobulin of each incubation which also contained 0.1 *M* potassium phosphate (pH 6.8). After 3 hr at 0–4°, the precipitated antigen-antibody complex was centrifuged and treated identically as in Figure 1.

enzyme for the kidney. The first slope of Figure 1 must be the result of the three species of antigen and antibody interaction. This hypothesis is supported by the shift of the first slope on immunoabsorption with the kidney antigen with no significant shift of the second slope. It also suggests that the ratios of antigen in the ammonium sulfate fraction are such that either the 2 mU/mg of immunoglobulin variety predominates in the liver or is the most weakly antigenic. Extrapolation of the immunotitration slopes to the abscissa makes it possible to compute equivalence values for each of the slopes. These values were compared with the equivalence ratios of antigen to antibody obtained from the double immunodiffusion and a table constructed for comparison (Table I). From this table it can be seen that there is excellent agreement between the immunodiffusion and immunotitration results of kidney PH.

Immunotitration and Immunodiffusion of Minimal Deviation Hepatoma (H4-II-E-C3) PH with Immunoglobulin. Preliminary experiments testing the stability of H4-II-E-C3 minimal deviation hepatoma (tissue culture) ammonium sulfate PH revealed the enzyme to be stable for 3 hr with no significant loss of activity when incubated at 0–4°. This period of incubation of the enzyme antibody mixture resulted in sufficient immunoprecipitation of the antigen-antibody complex to make a quantitative evaluation possible. Immunotitration of H4-II-E-C3 PH results in a single slope (Figure 6). No inhibition of PH activity was observed when control non-immune globulin was incubated at the maximum concentration of immunoglobulin. Extrapolation of the immunotitration slope to the abscissa results in an equivalence ratio of 2.06 mU of PH activity/mg of immunoglobulin. It is of interest that double immunodiffusion experiments (similar to those described in Figures 2 and 3), varying a 100-fold either the antigen or the immunoglobuline, did not result in an immunoprecipitin line. The reason for the lack of this immunoprecipitin line is under active investigation. Thus the behavior of H4-II-E-C3 is most similar to antigen 3 of liver, and suggests that it is also specific and does not cross-react with the two other antibodies of liver.

Discussion

Three immunologically distinguishable species of rat liver phenylalanine hydroxylase have been defined. Since the liver PH titration curves are the result of three separate antigen-antibody interactions, there can be no simple correlation of the first slope of immunotitration with the immunodiffusion results. Such a correlation would require individual isozyme-antigen titrations or, alternately, knowledge of the absolute quantity of antigen and its specific antibody in the titration experiments. Equivalence values represent ratios of antigen to antibody without any reference to the absolute quantity of a given antigen or its antibody.

In the case of kidney PH there is excellent agreement between the immunodiffusion and immunotitration results, indicating that the two conditions under which equivalence was determined were valid, and if the liver contained only a single antigen, similar results would have been observed. The single slope of the minimal deviation hepatoma H4-II-E-C3 phenylalanine hydroxylase immunotitration, with a near equivalence value of 2.01 mU/mg of immunoglobulin, is additional evidence for the same argument.

The strongest argument for the distinct immunological identity of at least two of the isozymes is derived from the immunoabsorption experiments. The kidney immunoabsorbed immunoglobulin distinctly lacks any residual antibody to react with kidney PH or antigen 2 of liver. Conversely, antibodies to liver PH antigens 1 and 3 do not cross-react with antigen 2 of liver or kidney PH.

The existence of three isozymes of rat liver PH of identical molecular weight but differing charge (Barranger et al., 1972), and the presence of two nonidentical subunits of 54,000 molecular weight (Gillam et al., 1974) would allow the theoretical consideration of three immunologically distinct isozymes of rat liver PH. If the nonidentical subunits represent α and β polypeptide chains, then it can be predicted that the three isozymes of liver PH would have the following structure: $\alpha\alpha$,$\alpha\beta\beta_L\beta_L$, and the one of kidney, $\beta_K\beta_K$. Alternately, if the subunits are identical (Kaufman and Fisher, 1970), then the isozymes could have the following structure: $\alpha\alpha$,$\beta\beta$,$\gamma_L\gamma_L$, indicating that each isozyme is coded for by a separate locus. Additionally it can be predicted, because of the large difference in equivalence values between the liver $\gamma_L\gamma_L$ (21 mU/mg of immunoglobulin) and kidney $\gamma_K\gamma_K$ (1.9 mU/mg of immunoglobulin), that the kidney isozyme is not identical with the liver with which it cross-reacts immunologically. It is no surprise that this difference cannot be detected on double immunodiffusion, where the immunoprecipitin lines of liver antigen 2 and kidney completely fuse. The relative insensitivity of immunodiffusion procedures in detecting structural changes is illustrated by carbonic anhydrase II proteins from a number of species (Tashian et al., 1968). Despite appreciable differences in amino acid composition, enzymes from humans, pig-tailed macaques, and green monkeys show complete immunological identity in the double-immunodiffusion technique if tested against antiserum to human enzyme. Similarly, Arnheim and Wilson (1967) were able to demonstrate differences in the lysozymes of birds by microcomplement fixation, but not by immunodiffusion.

A third possible mechanism by which isozymes may be generated is through the epigenetic modifications of protein structure by phosphorylation of serine residues, addition of carbohydrate groupings, and removal of components of the polypeptide chain by proteolytic enzymes. Characteristic sets of isozymes, the several members of which all appear to be products of a single allele, have been observed in studies of allelic variants of phosphoglucomutase (Harris, 1970).

The variation in antibody production to an identical antigenic challenge by different animals is the most likely explanation for the previous lack of identification of immunologically distinct isozymes of PH (Friedman et al., 1972; Ayling et al., 1974). Low and high affinity antibodies can alternate during the immune response (Macario and de Macario, 1973). Thus it is not unreasonable that there are differences in the antibody preparations by various investigators.

The two electrophoretic variants of purified rat liver PH were injected simultaneously to prepare antibody in sheep by Friedman et al. (1972). Immune serum without further purification was used for all of the immunological studies. No immunologically distinct isozymes were identified from this one preparation by a limited number of immunodiffusion and immunotitration experiments with purified enzyme and tissue extracts. The immunotitration studies were limited to the liver. The precipitated antigen-antibody complex was resuspended and its PH activity measured. The resuspended antigen-antibody complex lost 60% of its initial activity. This result suggests that the immune serum had an additional inhibitor which was not the immunoglobulin, or alternately, the enzyme was unstable under the conditions of testing. This inhibition accounts for greater than 60% of the total inhibition recorded at the maximum quantity of immune serum used (100 μl). In the present study, incubation of 9 mU of purified enzyme with 1 mg of immunoglobulin results in 61% of inhibition of enzyme activity within 4 hr at 0–4°. However, the resuspended antigen-antibody complex retained 92% of its initial activity. Ayling et al. (1974) similarly observed no inhibition of the antigen-antibody complex.

Antibody to PH, prepared by injecting rabbits with a purified preparation of rat liver PH which had only one of the electrophoretic variants, could not immunologically distinguish liver from kidney PH by immunotitration with immune serum (Ayling et al., 1974). The antigen-antibody complex required greater than 48 hr to precipitate and achieve a 50% inhibition of the initial activity. Because of the prolonged period required for antigen-antibody precipitation during which the enzyme was unstable, a differential precipitation of the antigen-antibody complex with 33% ammonium sulfate was used. Immunotitration of PH by this method did not distinguish liver from kidney PH (Ayling et al., 1974). In the present study, we were unable to quantitatively precipitate the immunoglobulin-enzyme complex with ammonium sulfate; 33% ammonium sulfate precipitated significant amounts of PH with nonimmune control globulin.

Further work to isolate and characterize each of these isozymes and their subunits, in addition to preparation of specific antibodies, would strengthen the evidence for the unique identity of the isozymes of phenylalanine hydroxylase.

Acknowledgments

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The Anomeric Specificity of Yeast Pyruvate Kinase toward Activation by D-Fructose 1,6-Bisphosphate†

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ABSTRACT: The anomeric specificity of D-fructose 1,6-bisphosphate activation of yeast pyruvate kinase has been investigated utilizing stopped-flow kinetics and synthetic analogs of D-fructose 1,6-bisphosphate. It has been demonstrated that the immediate product, β-D-fructose 1,6-bisphosphate, of the phosphofructokinase-catalyzed reaction increases the catalytic activity of the enzyme. Although exclusive activation by the α anomer is ruled out by these experiments the possibility that the allosteric site is anomerically nonspecific cannot be excluded owing to experimental limits. 2,5-Anhydromannitol 1,6-bisphosphate, 2,5-anhydroglucitol 1,6-bisphosphate, 1,6-diphosphohexitol, and methyl (α + β)-D-fructofuranoside 1,6-bisphosphate were tested as activators or inhibitors of the D-fructose 1,6-bisphosphate activation of pyruvate kinase. No activation was observed but inhibition of D-fructose 1,6-bisphosphate activation by 2,5-anhydromannitol 1,6-bisphosphate and 2,5-anhydroglucitol 1,6-bisphosphate was noted. Methyl (α + β)-D-fructofuranoside 1,6-bisphosphate and 1,6-diphosphohexitol also proved to inhibit weakly. The collective data suggest that the allosteric site on yeast pyruvate kinase may be nonspecific with respect to anomeric configuration, but that a C-2 hydroxyl is necessary for activation by D-fructose 1,6-bisphosphate.

Pyruvate kinase catalyzes one of the rate-limiting steps in glycolysis (Scrutton and Utter, 1968). The kinases found in liver and yeast are subject to feed forward activation by D-fructose-1,6-P_2[1] (Kane, 1973). Since D-fructose-1,6-P_2 exists in solution in three rapidly equilibrating configurations, it became of interest to investigate whether pyruvate kinase from yeast is subject to stereospecific activation by only one of the three possible forms. Several other glycolytic and gluconeogenic enzymes have been observed to show a dependence on the anomeric nature of their substrates; these include phosphoglucose isomerase (Schray et al., 1973), D-fructose-6-P kinase (Fishbein et al., 1974; Wurster and Hess, 1974a; Bar-Tana and Cleland, 1974; Koerner et al., 1974), fructose bisphosphatase (Benkovic *et al.,* 1974), and aldolase (Schray et al., 1975; Wurster and Hess, 1974b). This manuscript presents the results of experiments utilizing stopped-flow kinetic techniques and D-fructose-1,6-P_2 analogs relevant to the question of anomeric specificity.

Experimental Section

Materials. Phosphoenolpyruvate, ADP, ATP, NADH, D-fructose-1,6-P_2, D-fructose-6-P, and $NADP^+$ were all obtained from the Sigma Chemical Co. Lactic dehydrogenase, specific activity 815 units/mg, and D-fructose-6-P kinase, specific activity 170 units/mg, were also obtained from Sigma.

Pyruvate kinase was isolated from fresh Budweiser bakers yeast obtained from Anheuser Busch Inc., Baltimore, Md., according to the procedure of Hunsley and Suelter (1969) resulting in a preparation with a specific activity of 375 units/mg. Pyruvate kinase activity was measured at pH 6.2 utilizing the lactic dehydrogenase enzyme couple.

2,5-Anhydromannitol-1,6-P_2, 2,5-anhydroglucitol-1,6-P_2, and 1,6-diphosphohexitol were previously prepared according to published procedures (Benkovic et al., 1971).

Methods. Stopped-flow studies were performed on a Durrum Gibson stopped-flow spectrophotometer at 25° and 340 nm. The spectrophotometer is designed to have a mixing ratio of 1:1 in a minimum volume of approximately 0.15 ml; a total dead time of 10 msec was estimated by mixing 0.01 *M* $Fe(NO_3)_3$ in 0.1 *N* H_2SO_4 with 0.01 *M* KCNS and following the appearance of product at 650 nm. Reactions were started by mixing an equal volume of 0.1 *M* Tris (pH 7.5), 0.025 *M* $MgCl_2$, 0.1 *M* KCl, 2 m*M* ADP, 2 m*M* P-

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[1] The abbreviations P_2 and P will designate bisphosphate and monophosphate, respectively.