PRODUCTION OF ANTIBODIES TO SHEEP LIVER DIHYDROPTERIDINE REDUCTASE: CHARACTERIZATION AND USE TO STUDY THE ENZYME DEFECT IN A VARIANT FORM OF PHENYLKETONURIA

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SUMMARY - An antiserum to sheep liver dihydropteridine reductase has been prepared in rabbits. The antiserum cross-reacts with dihydropteridine reductases from human, rat and bovine tissues. Using this antiserum, it was not possible to detect any cross-reacting material in the liver of a phenylketonuric child whose genetic defect has been shown to be due to a lack of detectable dihydropteridine reductase activity.

INTRODUCTION - The mammalian phenylalanine hydroxylase system is complex, consisting of at least two enzymes, phenylalanine hydroxylase and dihydropteridine reductase, and two cofactors, tetrahydrobiopterin and a reduced pyridine nucleotide (1).

In all cases of phenylketonuria examined in detail, the affected component of the system has been phenylalanine hydroxylase (2). However, it has been predicted (3) that phenylketonuria could also be caused by a deficiency in one of the other components of the system, tetrahydrobiopterin or dihydropteridine reductase. This type of variant would lead to symptoms of classical phenylketonuria, i.e., elevated serum phenylalanine levels, inability to metabolize a load of phenylalanine, and ultimately, mental retardation. Since these components are also essential for the biosynthesis of the neurotransmitters, dopamine (and norepinephrine)(4) and serotonin (5), it could be anticipated that an individual lacking either tetrahydrobiopterin or dihydropteridine reductase would not only have the symptoms of classical phenylketonuria, but would also show some neurological symptoms. Furthermore, these neurological symptoms could not be expected to be controlled by restriction of phenylalanine intake.

Recently, a baby who had been diagnosed as a classical phenylketonuric and who had progressive neurological deterioration despite excellent dietary control of his serum phenylalanine levels (6,7), was found to have relatively unaffected levels of all the components of the phenylalanine hydroxylase system except for a complete lack of measurable dihydropteridine reductase activity. In this paper we present details of the preparation of a specific antiserum to sheep liver dihydropteridine reductase, its characterization, and the results of its application to the study of this case of dihydropteridine reductase deficiency.

MATERIALS AND METHODS - Dihydropteridine reductases were purified from sheep, human and rat livers as previously described (8). Complete Freund's adjuvant was from Difco. Horseradish peroxidase was from Sigma. Dihydrofolate reductase, purified from beef liver by affinity chromatography was a gift from Dr. B. T. Kaufman, NIAMDD. 6,7-Dimethyltetrahydropterin was synthesized in this laboratory (9).

Assay of dihydropteridine reductase - Dihydropteridine reductase activity was measured by following the reductase-dependent oxidation of DPNH spectrophotometrically upon generation of quinonoid 6,7-dimethyl-dihydropterin from 6,7-dimethyl-tetrahydropterin with horseradish peroxidase and H_2O_2 at 25° (8).

Assay of dihydrofolate reductase - Purified beef liver dihydrofolate reductase activity was measured by the method of Osborne and Huennekens (10) using dihydrofolic acid freshly prepared by the method of Futterman (11).

Preparation of antigen and immunization - Human liver dihydropteridine reductase has previously been obtained in a high degree of purity in this laboratory (8). However, not enough of this material was available for production of an antiserum. Therefore, we decided to prepare an antiserum against highly purified sheep liver dihydropteridine reductase, which can be relatively easily purified in large amounts, in the hope that it would cross-react with the human enzyme.

Peak Sephadex G-100 fractions of sheep liver dihydropteridine reductase (~80% pure by disc gel electrophoresis), were concentrated and subjected to disc gel electrophoresis (8). Approximately 1.5 mg of the enzyme fraction was applied to 10 gels. The gels were subjected to electrophoresis at 4° until the tracking dye had migrated 5 cm. One gel was stained with Coomassie blue for 30 minutes at 40° and then destained for 30 minutes at 40°. The corresponding enzyme band on each gel (R_f = 0.33-0.35) was then cut out with a razor blade, and the gel segments pooled and homogenized in 1 ml of 0.15 M KCl containing 10 mM Tris-HCl, pH 7.4, and 1 ml Freund's complete adjuvant. Rabbits were injected subcutaneously on the back at multiple sites with 0.2 ml per site. Control rabbits were injected with the corresponding gel areas pooled from blank gels. Rabbits were injected two times, one week apart. Ten days after the last injection, the animals were bled from the ear vein, the blood allowed to clot, and the serum collected by centrifugation.

RESULTS AND DISCUSSION - The antiserum to sheep liver dihydropteridine reductase produced in rabbits cross-reacts with dihydropteridine reductases

from a wide range of species and tissues. In Figure 1. are shown the results of a double-diffusion experiment testing the antiserum to pure sheep liver dihydropteridine reductase against dihydropteridine reductase at various steps of the purification. In all cases, a single precipitin line was observed. No lines were seen when control serum was used in place of the antiserum.

In Figure 2. are shown the results of an immunodiffusion experiment

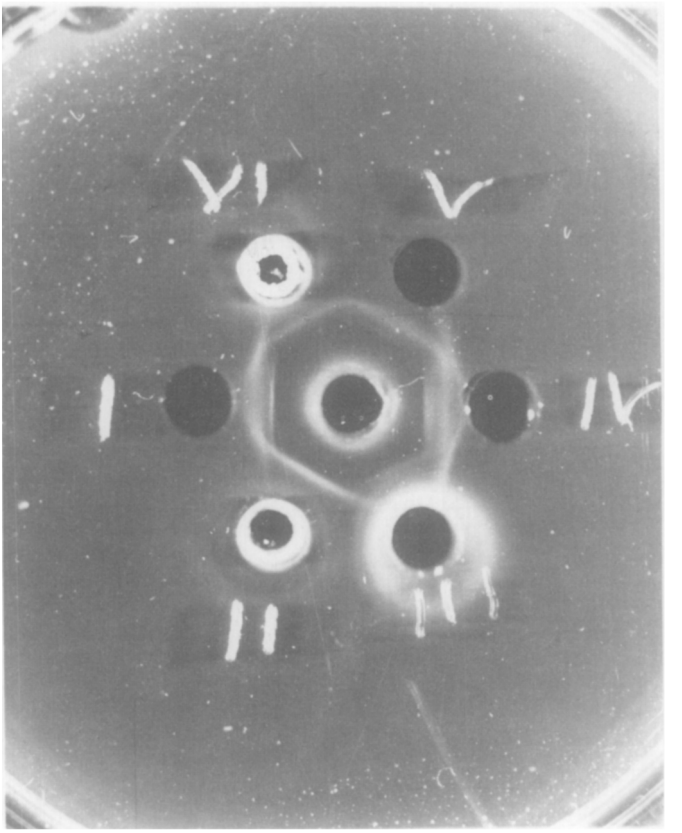

Fig. 1. Immunodiffusion experiment with various stages of purification of sheep dihydropteridine reductase. The center well contained 5 µl of undiluted antiserum. Well I contained 2 µl of DEAE peak fraction (4.7 mg/ml). Well II contained 5 µl of calcium phosphate fraction (2.5 mg/ml). Well III contained 10 µl from the alkaline ammonium sulfate fraction (27.4 mg/ml). Well IV contained 15 µl from the zinc-alcohol step (35.3 mg/ml). Well V contained 15 µl from the first ammonium sulfate fractionation (34.3 mg/ml). Well VI contained 20 µl from the sheep liver extract (25.6 mg/ml). In all cases, the volume per well was adjusted to 20 µl by the addition of 0.15 M KCl. The photograph was taken after 24 hours at 4°.

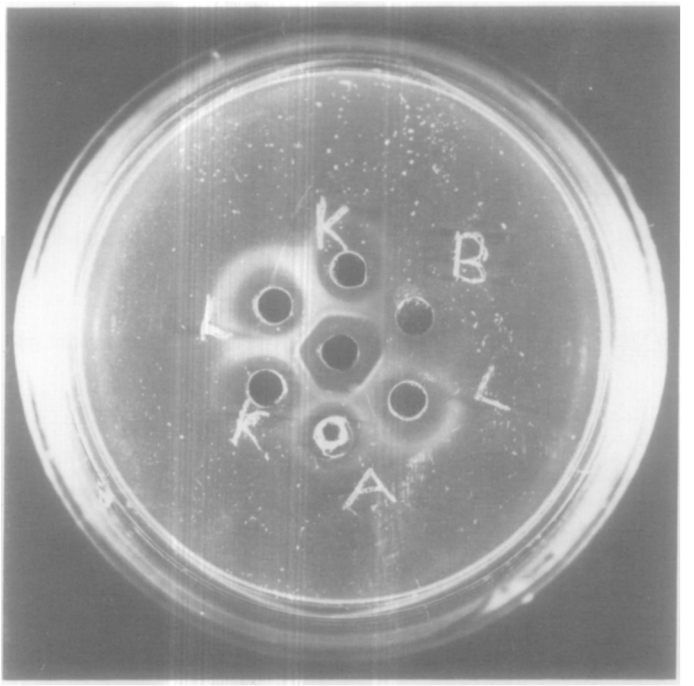

Fig. 2. Immunodiffusion experiment with dihydropteridine reductases purified from bovine liver, kidney, adrenal gland and brain cortex. Dihydropteridine reductases were purified through the first ammonium sulfate step (8) and taken up in 0.1 volume of 10 mM Tris-HCl, pH 7.4. The volumes per well were: antiserum (center well), 10 µl; liver (L), 5 µl; kidney (K), 5 µl; brain cortex (B), 15 µl; adrenal gland (A), 15 µl. Specific activities in µmoles DPNH oxidized/min/mg protein and protein concentrations in mg/ml were: liver 0.22 and 105; kidney, 0.26 and 82; adrenal, 0.05 and 85; cortex, 0.03 and 43. The volume per well was adjusted to 15 µl by the addition of 0.15 M KCl. The plate was incubated for 24 hours at 4°. Control serum gave no precipitin lines.

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testing the rabbit anti-sheep dihydropteridine reductase against fractionated extracts of various bovine tissues that are known to contain dihydropteridine reductase: liver, kidney, adrenal medulla, and brain cortex. In all cases a single precepitin line was observed indicating that the enzymes in all these tissues share common antigenic determinants.

The antiserum is more effective in inhibiting sheep dihydropteridine reductase than the enzyme from other species. In Figure 3. are shown the results of a quantitative immunotitration of dihydropteridine reductase purified from sheep, human and rat livers. As can be seen, the antiserum is

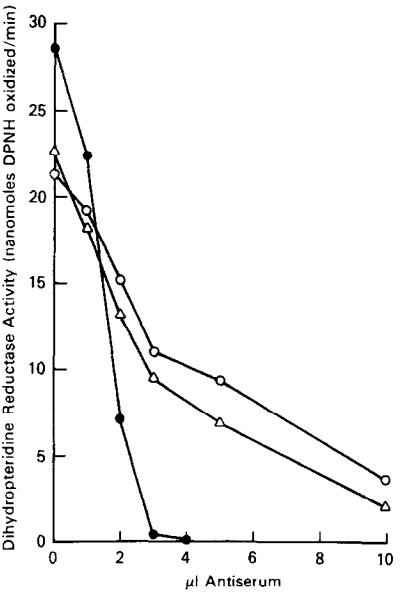

Fig. 3. Immunotitration of sheep, rat, and human liver dihydropteridine reductases. Sheep liver dihydropteridine reductase purified through the calcium phosphate step (8)(0.027 mg) ●---●, rat liver dihydropteridine reductase purified through the alkaline ammonium sulfate step (8) (0.2 mg) o---o and human liver dihydropteridine reductase purified through the Sephadex G-100 step (8) (approximately 80% pure, 0.004 mg) Δ---Δ were incubated with the indicated volumes of rabbit antiserum in a total volume of 0.11 ml for 4 hours at 4°. Control rabbit serum was added to keep the total volume of serum at 10 μl. Aliquots were removed after centrifugation at 8000 X g for 2 minutes and the supernatant dihydropteridine reductase activity remaining was measured as described in Methods. Incubations with control rabbit serum alone had no effect on enzyme activity (data not shown).

more then twice as effective against the sheep enzyme than it is against the rat or human enzyme.

In the presence of 7,8-dihydrobiopterin, the enzyme, dihydrofolate reductase, is an essential component of the phenylalanine hydroxylase system (12). The biosynthesis of the natural cofactor for phenylalanine hydroxylase, tetrahydrobiopterin, may require dihydrofolate reductase. In addition, dihydrofolate reductase may act as a scavenger for any quinonoid dihydrobiopterin that escapes reduction by the dihydropteridine reductase-catalyzed reaction and rearranges to 7,8-dihydrobiopterin. Since the reactions catalyzed by both reductases are very similar, it was of interest to test the

antiserum to dihydropteridine reductase against dihydrofolate reductase. By immunodiffusion no detectable precipitin lines were seen with amounts of dihydrofolate reductase up to 20 μg per well, whereas a sharp line could be seen with partially purified bovine liver dihydropteridine reductase (FIG 2). In addition, no inhibition of dihydrofolate reductase activity was seen when 20 μg of dihydrofolate reductase were incubated for 4 hours at 4° with from 5 to 100 μl of antiserum. Thus, bovine dihydrofolate reductase and dihydropteridine reductase do not share any antigenic properties.

The cross-reactivity of the anti-sheep dihydropteridine reductase rabbit serum with human dihydropteridine reductase has allowed us to investigate the nature of the defect in a baby with no detectable liver or brain dihydropteridine reductase activity. When an extract from a liver biopsy from this patient was tested by immunodiffusion against the antiserum, no detectable cross-reacting material was found. An amount of dihydropteridine reductase equal to 10% of that present in an extract from normal liver could have been detected. Although the results of the immuno-diffusion experiments confirm and extend our previous enzyme assay results, i.e., that the patient lacks dihydropteridine reductase activity, they still leave open many possibilities for explaining the genetic basis of his enzyme lack. The deficiency could be due to: a) a deletion mutation in which no gene product is formed; b) a regulatory mutation, in which markedly decreased amounts of the normal gene product are produced; c) or a structural gene mutation, in which an altered gene product is produced which does not cross-react with the antiserum or which is degraded so rapidly that its concentration is below our limits of detection. Further studies will be required to discriminate between these possibilities.

REFERENCES

1. Kaufman, S. (1971) Advan. Enzymol. 35, 245-319.
2. Kaufman, S. (1958) Science, 128, 1506-1508.
3. Kaufman, S. (1967) Proc. of Conf. on Treatment of Phenylketonuria & Allied Diseases, Anderson, J.A., and Swaiman, K.F., eds., Washington, D.C.. Government Printing Office, p.205.
4. Shiman, R., Akino, M., and Kaufman, S., (1971) J. Biol. Chem., 246, 1330-1340.

5. Friedman, P.A., Kappelman, A.H., and Kaufman, S. (1972) J. Biol. Chem., 247, 4165-4173.
6. Butler, I.J., Holtzman, N.A., Kaufman, S., Koslow, S.H., and Milstien, S., (1975) Pediatric Res. 9, 348.
7. Kaufman, S., Holtzman, N.A., Milstien, S., Butler, I.J., and Krumholz, A. (1975). Submitted for publication.
8. Craine, J.E., Hall, E.S., and Kaufman, S. (1972) J.Biol. Chem. 247, 6082-6091.
9. Mager, H.I.X., Addink, R., and Berends, W. (1967) Rec. Trav. Chim., 186, 833-851.
10. Osborne, M.J., and Huennekens, F.M., (1958) J.Biol. Chem., 233, 969-974.
11. Futterman, S., (1957) J. Biol. Chem. 228, 1031-1038.
12. Kaufman, S., (1967) J. Biol. Chem., 242, 3934-3943.