IMMUNOCHEMICAL DETECTION AND CHARACTERISTICS OF SUBUNIT COMPOSITION OF HUMAN BRAIN PHENYLALANINE HYDROXYLASE

V. V. Chestkov and A. V. Laptev

UDC 612.822.1.015.1:577.152.199.1].088.1.083.33+616.831-008.931:577.152.199.1]-078.73

KEY WORDS: phenylalanine hydroxylase; human brain; immunochemistry; subunits.

Detection of tissue-specific enzymes in very small quantities in organs in which they have not previously been found is an important step toward the understanding of pathways of metabolism in the human body and provides a basis for the study of regulation of gene expression in various tissues. Investigations of this kind are based on the introduction of ever-more sensitive methods of detection of concrete proteins [1, 2, 5].

Phenylalanine hydroxylase is an enzyme, the inherited defects of which lead to the widespread disease known as phenylketonuria. The presence of the enzyme in the liver and kidneys only has been established by determination of its activity in these organs [13]. Although there are indications of the presence of immunoreactive material in brain tissue [6], the structural characteristics of the antigen thus discovered have not so far been described.

The aim of this investigation was the immunochemical detection of phenylalanine hydroxylase-like antigen in brain tissue and determination of its structural characteristics, in order to identify the antigen.

EXPERIMENTAL METHOD

Liver biopsy was carried out during operations for liver trauma and for diagnostic purposes. Cadaveric organs were obtained 2-3 h after death of patients with acute heart failure. The material obtained was transported in dry ice and stored at −70°C.

Brain and liver extracts were obtained by centrifugation of the tissue homogenate at 105,000g [3]. Activity of phenylalanine hydroxylase and the concentration of the protein in the samples were measured as described previously [3]. To determine the antigen quantitatively, rocket immunoelectrophoresis was used with a 1% solution of antiserum to phenylalanine hydroxylase in the gel. Phenylalanine hydroxylase purified by affinity chromatography on phenyl-sepharose [3] was used as the standard.

Electrophoresis of proteins was carried out by the method in [9] and two-dimensional electrophoresis by the method in [12]. The gels were stained with silver [11]. To detect phenylalanine hydroxylase after electrophoresis by immuno-blotting, the IgG fraction of the antisera, conjugated with horseradish peroxidase in a molar ratio of 1:2, was used. Proteins were transferred to a nitrocellulose filter overnight with a field voltage gradient of 9 V/cm [15]. The nonspecific binding sites on the filter were covered by incubation in 50% nonimmune rabbit serum with 1% Tween-80. The filter was then incubated in the same solution with the addition of the conjugate in a concentration (as immunoglobulins) of 0.1 mg/ml. The sites of localization of the antigen were detected on treatment of the filter with a solution containing 0.3 mg/ml of 3,3'-diaminobenzidine in 0.005% H_2O_2 and 0.05M Tris-HCl (pH 7.25).

Immunosorbent for phenylalanine hydroxylase was obtained by binding BrCN-sepharose with IgG of the antisera. To isolate phenylalanine hydroxylase, solutions containing Mg protein of liver extract or 100 mg protein of brain extract were applied to a column with 2 ml of the synthesized sorbent overnight. The immunosorbent was washed with a solution of 0.05 M Tris-HCl (pH 7.25) with 0.5M NaCl and the antigen was eluted with 0.05M triethanolamine (pH 12.5) with 0.15M NaCl. The eluate was collected in fractions and the pH of the samples adjusted

Institute of Medical Genetics, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR N. P. Bochkov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 106, No. 7, pp. 30-34, July, 1988. Original article submitted October 23, 1987.

0007-4888/88/0007-0949$12.50

TABLE 1. Concentration of Phenylalanine Hydroxylase Antigen in Autopsy Specimens of Human Organs (in μg/g tissue)

Patient No.	Liver	Brain
1	1920	27
2	1520	37
3	1560	20

Legend. Concentration of antigens determined from a calibration curve obtained with affinity-purified phenylalanine hydroxylase [3].

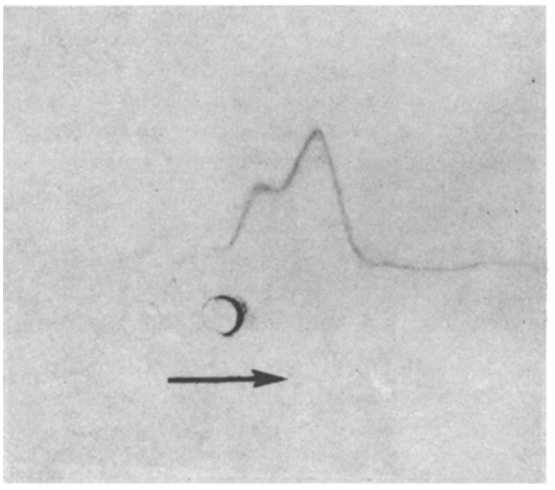

Fig. 1

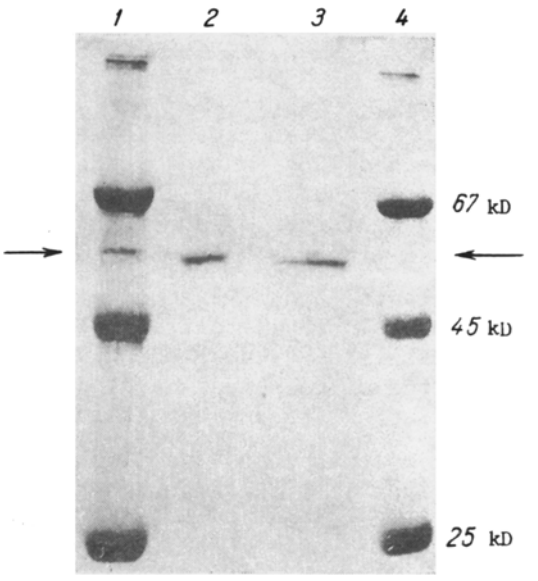

Fig. 2

Fig. 1. Cross-immunoelectrophoresis of proteins of brain and liver extracts with antiserum to human liver phenylalanine hydroxylase. Liver extracts (4 μg protein) and brain extract (40 μg protein) introduced into gel 4 mm apart and electrophoresis carried out in direction 1 (direction of protein migration indicated by arrow). After electrophoresis in direction 2, the gel was stained to reveal the precipitation line. The left arm of the precipitation line was formed by antigen from brain extract, the right arm by liver antigen.

Fig. 2. Electrophoresis of proteins purified on immunosorbent to human liver phenylalanine hydroxylase. The following marker proteins were applied in a dose of 5 μg to lanes 1 and 4: bovine serum albumin (67 kD), ovalbumin (45 kD), and chymotrypsinogen A (25 kD). Proteins (3 μg) from human liver extract, purified on immunosorbents, applied to lane 2; proteins (3 μg) from brain extract of patient No. 1, purified on immunosorbent, applied to lane 3. Components with electrophoretic mobility of human liver phenylalanine hydroxylase indicated by arrow.

to 7.5 by addition of 1M Tris-HCl (pH 7.0). The composition of the proteins was verified electrophoretically.

EXPERIMENTAL RESULTS

An antigen identical with the antigen from liver biopsy specimens was found by immunoelectrophoresis in extracts of human liver autopsy material. The investigation could accordingly be carried out on autopsy organs and the phenylalanine hydroxylase antigen assayed by rocket immunoelectrophoresis (Table 1).

Antigen was also found by this method in brain extract. That it was identical with the liver antigen was confirmed by co-immunoelectrophoresis of the liver and brain extracts (Fig. 1). However, the concentration of phenylalanine hydroxylase-like antigen in the brain was lower than in the liver, by almost 60 times (Table 1).

An enzymically active protein with specific activity of 20 nmoles/min·mg protein was purified from the extract of liver biopsy material on immunosorbent to phenylalanine hydroxylase. This activity is close to the specific activity of phenylalanine hydroxylase in extracts

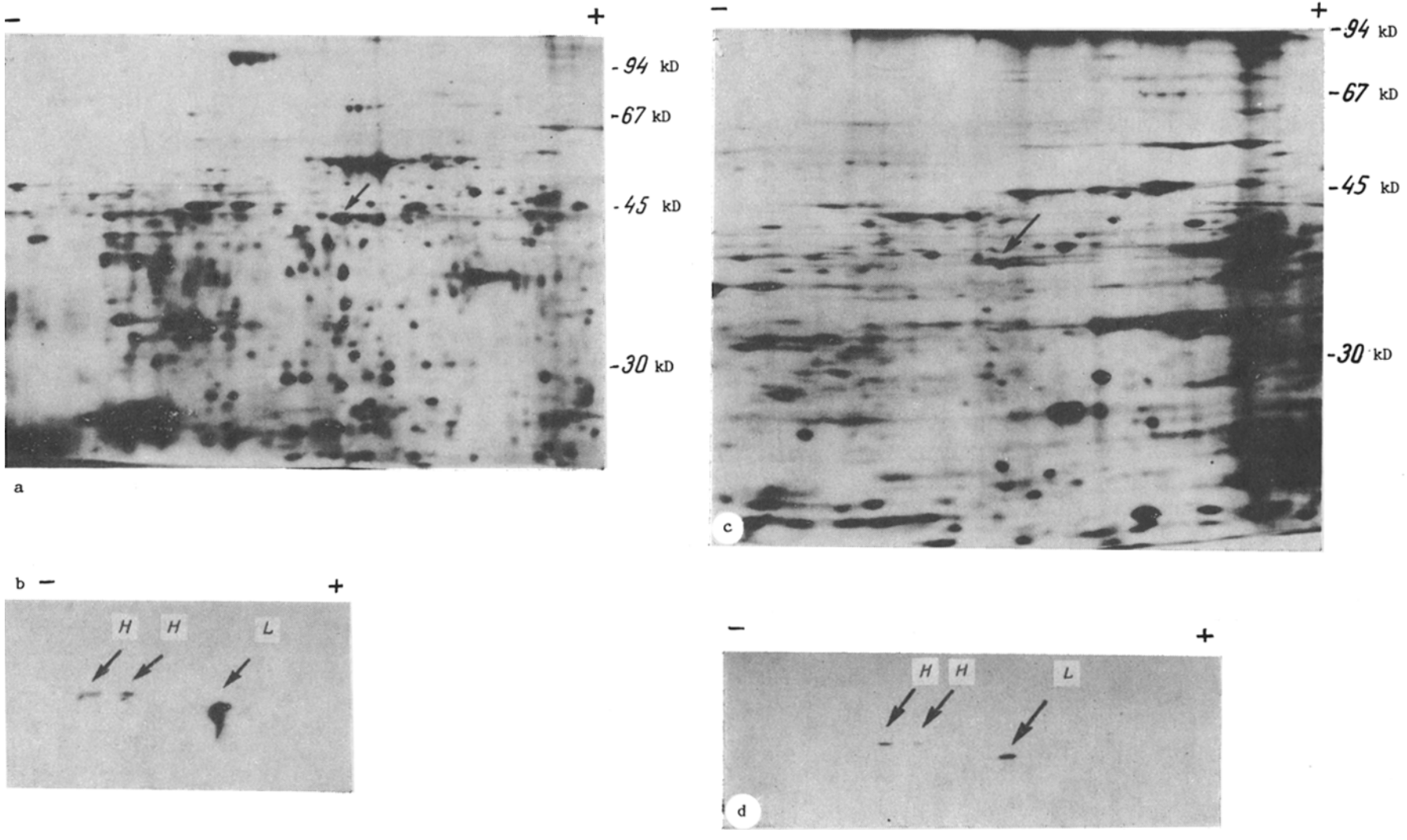

Fig. 3. Characteristics of subunitary composition of phenylalanine hydroxylase of human liver and brain extract revealed by two-dimensional electrophoresis (100 μg protein of organ extract was applied to the gel). a) Two-dimensional electrophoresis of liver extract proteins (spot disappearing from liver extract after passage through immunosorbent to phenylalanine hydroxylase indicated by arrow); b) immunoblotting of liver extract after two-dimensional electrophoresis (arrows indicate two H subunits and one L subunit of phenylalanine hydroxylase); c) two-dimensional electrophoresis of brain extract proteins (arrow indicates spot coinciding in position with L-subunit of antigen, revealed by immunoblotting); d) immunoblotting of brain extract proteins after two-dimensional electrophoresis (arrows indicate two H subunits and one L subunit of antigen).

of liver biopsy material (19.0 ± 0.3 nmoles/min·mg protein). Profile elution of a protein with a molecular weight of 55 kD from the immunosorbent (Fig. 2), subjected to denaturing electrophoresis, showed the same level of enzymic activity as was found in the samples. Since the molecular weight of the phenylalanine hydroxylase subunit is 55 kD [3, 4], it can be concluded that a highly purified and active human liver phenylalanine hydroxylase had been obtained.

A protein with electrophoretic mobility the same as that of liver phenylalanine hydroxylase was obtained by this method from extracts of autopsy brain material (Fig. 2). This protein had no enzymic activity, probably because phenylalanine hydroxylase is an unstable enzyme, losing its activity 1-2 h after death [3, 10].

The coincidence of the two independent characteristics of the protein, its immunochemical properties and the molecular weight of its subunits, suggested that the same protein is found in all tissues. However, there is evidence [6, 7] that tyrosine hydroxylase and tryptophan hydroxylase also give a cross reaction with antibodies to phenylalanine hydroxylase. To confirm the identical nature of the liver and brain antigen, it was therefore mapped by two-dimensional electrophoresis of proteins in extracts of these organs.

Spots formed by the antigen during two-dimensional protein electrophoresis were localized by comparative analysis of proteins of the liver extract before and after immunosorption of the enzyme on the immunosorbent. A sharp change was found in the amount, or even its complete absence, after immunosorption of one spot with molecular weight of 55 kD (Fig. 3a, arrow), isofocusing in the same region as albumin, was found.

The subunitary composition of phenylalanine hydroxylase was determined after immunoblotting of the gels obtained by two-dimensional electrophoresis of liver extract proteins (Fig. 3b). The principal (L) subunit of the enzyme was thus revealed, and its position coincided with the spot of the enzyme localized in Fig. 3a. Two minor H subunits, differing from each other in pI, and from the L subunit in pI and molecular weight (57 kD), also were found. There are also certain differences in isofocusing of the antigen in liver extracts compared with the purified enzyme [14].

Because phenylalanine hydroxylase constituted a smaller proportion of the proteins of brain extract, the only method capable of localizing its subunits on the gel after two-dimensional electrophoresis of brain proteins was that of protein immunoblotting (Fig. 3c, d). Just as in the case of liver, three subunits of the enzyme were found among proteins of the brain extract: two H subunits and one L subunit. Their coordinates on two-dimensional electrophoresis coincided with those of the enzyme from liver extracts, and during coelectrophoresis they were located at identical points.

These data show that a protein with the same molecular weight, subunitary composition, and immunochemical properties is found in brain and liver extracts. Since the antibodies used adsorb active phenylalanine hydroxylase in liver extract, these results suggest that the antigen discovered is phenylalanine hydroxylase protein.

The use of high-resolution methods of tyrosine detection has revealed phenylalanine hydroxylase activity in rat brain [8]. However, the nature of this activity is not yet clear, for the enzyme responsible for its manifestation has not been discovered. In the present investigation a probable carrier of this activity was found in human brain.

LITERATURE CITED

1. G. I. Abelev, Byull. Éksp. Biol. Med., 76, No. 11, 122 (1973).
2. T. P. Klyushnik, S. G. Voloshchuk, M. V. Aksenova, and G. Sh. Burbaeva, Byull. Éksp. Biol. Med., 99, No. 2, 179 (1985).
3. V. V. Chestkov, A. V. Laptev, E. M. Danilina, and S. S. Shishkin, Biokhimiya, 51, No. 7, 1214 (1986).
4. J. -P. Abita, F. Blandin-Savoja, and F. Rey, Biochem. Int., 7, 727 (1983).
5. B. Bloch, T. Popovici, M. J. Levin, et al., Proc. Natl. Acad. Sci. USA, 82, 6706 (1985).
6. R. G. H. Cotton, H. M. Dahl, J. F. B. Mercer, et al., J. Inher. Metab. Dis., 9, Suppl. 2, 206 (1986).
7. P. F. Friedman, T. Loyd, and S. Kaufman, Molec. Pharmacol., 8, 501 (1972).
8. S. Ishimitsu, S. Fujimoto, and A. Ohara, Chem. Pharm. Bull., 33, 3887 (1985).
9. U. K. Laemmli, Nature, 227, 680 (1970).
10. C. Mitoma, R. Auld, and S. Udenfriend, Proc. Soc. Exp. Biol. (New York), 94, 634 (1957).
11. J. H. Morrisey, Anal. Biochem., 117, 307 (1981).
12. P. H. O'Farrell, J. Biol. Chem., 250, 4007 (1975).

13. C. R. Scriver and C. L. Clow, Ann. Rev. Genet., 14, 179 (1980).
14. S. C. Smith, B. E. Kemp, W. J. McAdam, et al., J. Biol. Chem., 259, 11284 (1984).
15. H. Towbin, T. Staehelin, and J. Gordon, Proc. Natl. Acad. Sci. USA, 76, 4350 (1979).

LIVER MONO-OXYGENASE SYSTEM FUNCTION IN EXPERIMENTAL MYOCARDIAL INFARCTION

O. R. Grek, Yu. I. Borodin,
V. I. Sharapov, A. A. Zykov,
and T. A. Rybakova

UDC 616.127-005.8-092.9-07:
616.36-008.931.577.152.1

KEY WORDS: myocardial infarction; liver mono-oxygenase; lipid peroxidation.

Changes in the structure and functions of the liver in myocardial infarction have been the subject of much research [3, 6]. However, changes in the mono-oxygenase system of the liver, responsible for metabolic conversions of cholesterol [7], saturated and unsaturated fatty acids [14], steroid hormones [9], and most drugs [5], in myocardial infarction have not yet been studied.

The aim of this investigation was to study the functional activity and content of enzymes of the liver mono-oxygenase system in experimental myocardial infarction, in its early and late stages.

EXPERIMENTAL METHOD

Experiments were carried out on 100 male albino rats weighing 200-240 g. There were two series of experiments. In series I the animals were anesthetized with ether, thoracotomy performed, the pericardium incised, and the left descending coronary artery ligated. In series II thoracotomy was performed and the pericardium incised but the coronary artery was not ligated (mock operation). Intact rats kept under standard animal house conditions served as the control. The animals were decapitated on the 1st, 3rd, 7th, 14th, and 21st days after the operation, the liver washed out with physiological saline, and the microsomal fraction was isolated by differential centrifugation [1]. The velocity of N-demethylation of aminopyrine and of p-hydroxylation of aniline, and concentrations of cytochromes P-450 and b_5 in the microsomes were determined as described previously [11]. The concentration of the microsomal cytochromes was recorded on a Hitachi-356 double-beam spectrophotometer (Japan). Activity of spontaneous lipid peroxidation (LPO) of the microsomal membranes was estimated from the malonic dialdehyde (MDA) concentration in the microsomal fraction [2] on the 1st, 7th, and 21st days after the operation. The protein concentration in the microsomes was determined by the method in [13]. Differences between the mean values compared were considered significant at the $p<0.05$ level (Student's t test).

EXPERIMENTAL RESULTS

The acute period (1st-3rd days) after the operation was characterized by inhibition of function and by a fall in the concentrations of the principal microsomal mono-oxygenases. In animals undergoing the mock operation, the fall in the velocity of N-demethylation of aminopyrine and of p-hydroxylation of aniline was maximal (by 49.6 and 32.4% respectively) on the 1st day, after which levels of activity of the parameters of microsomal metabolism were gradually restored on the 7th day after the operation (Table 1). A quantitative study of microsomal cytochromes P-450 and b_5 also revealed a significant decrease in the acute period after the operation, followed by recovery to the initial level on the 7th day.

The time course of changes in microsomal mono-oxygenase activity in the group of animals with occlusion of the coronary artery differed in character. The velocity of N-demethylation

Novosibirsk Medical Institute. Institute of Physiology, Siberian Branch, Academy of Medical Sciences of the USSR, Novosibirsk. Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 106, No. 7, pp. 34-37, July, 1988. Original article submitted July 13, 1987.

0007-4888/88/0007-0953$12.50