Brain-Specific Proteins: Solid-Phase Immunobioluminescence Assay for Neuron-Specific Enolase in Human Plasma

K.-D. Gerbitz, J. Sommer, and J. Thallemer

We raised specific antibodies in rabbits against pure αα- or γγ-isoenzymes of enolase (EC 4.2.1.11) purified from human brain. After being specifically immunoabsorbed by the respective Sepharose-coupled enolase isoenzymes, the purified antibodies are desorbed by acidification and coated onto polystyrene tubes. Each sample containing the isoenzyme activity to be determined is incubated in the respective coated tube. The coating is then washed, and the reaction of the antibody-bound enzyme is initiated by adding 2-phosphoglycerate, ADP, and pyruvate kinase. The accumulation of ATP is measured by following the increase in light emission in the firefly luciferase bioluminescence system. The assay is as specific as the antibody used for coating. Its detection limit is about 5 × 10⁻⁹ U, corresponding to about 0.1 pg or 10⁻¹⁸ mol of enzyme protein per assay. Activities of enolase isoenzymes in human plasma can be evaluated separately, rapidly, and precisely. We used the assay to measure enolase isoenzyme activities in plasma of patients suffering from different types of malignant tumors.

Additional Keyphrases: enolase isoenzymes · bioluminescence · tumor marker · cancer · luciferase · assessing and monitoring disease · reference interval

The γγ-isoenzyme of enolase (2-phospho-D-glycerate hydrolyase; EC 4.2.1.11), the so-called neuron-specific enolase, is reportedly a key enzyme in the amine precursor uptake and decarboxylation cell system (1). Recently the enzyme was detected in some endocrine tumors (2) and in serum of patients suffering from small-cell cancer of the lung (3, 4), and was used to assess the extent of disease and the response to therapy. Its activity in serum is too low for the usual photometric tests to detect the enzyme. Furthermore, serum not only contains the γγ-isoenzyme, but also other enolase isoenzymes such as αα, αγ, and the isoenzyme ββ of muscle origin.1 Therefore, measurements of γγ-enolase in serum must be both highly specific and sensitive. Enzyme immunoassay and RIA methods have been devised (4–8), but are rather cumbersome for routine use.

Here we report a new type of assay, in which the specificity of purified antibodies coupled to a solid phase is combined with the sensitivity of the firefly bioluminescence system.

Materials and Methods

Materials

Dowex 1- × 8, 200–400 mesh, was purchased from Bio-Rad Laboratories, CNBr-activated agarose (Sepharose) from Pharmacia. The polystyrene tubes (0.8 × 4 cm) to be coated with antibody were products of Greiner, Labortechnik, 7440 Nürtingen, F.R.G. The ATP-monitoring reagent (LKB-Wallac, Turku, Finland) was used according to the supplier's instructions. 2-Phosphoglycerate, pyruvate kinase (EC 2.7.1.40; 200 kU/g of protein), and ATP were products of Boehringer GmbH, Mannheim, F.R.G. All other chemicals were from Merck, Darmstadt, F.R.G., and were of analytical grade.

Procedures

Photometry of enolase was done according to Bergmeyer et al. (9). Creatine kinase (EC 2.7.3.2) isoenzyme BB was assayed according to the recommendations of the German Society for Clinical Chemistry (10). The ADP was contaminated with ATP, so we chromatographed it on Dowex 1- × 8 as described by Lundin (11). Protein was determined either by measuring the absorbance at 280 nm or by the method of Lowry et al. (12). Bioluminescence was measured at 25°C with a luminometer (Bioluminomat LB 9500 T; Berthold, 7847 Wildbad, F.R.G.) coupled to a Servogor recorder RE 511.

General

Isoenzyme preparation. We prepared αα- and γγ-enolase isoenzymes from human brain by conventional methods (13–15). Physicochemical characterization showed the products to be pure and homogeneous, with very similar relative molecular masses, 87 000 for αα-enolase and 86 000 for γγ-enolase, but with strikingly different charges. The isoelectric point of αα-enolase was 8.2, that of γγ-enolase 4.7. These results are consistent with reports in the literature (13, 14, 16, 17). Because of similarities in charge and size, creatine kinase isoenzyme BB co-chromatographs with γγ-enolase in the preparation procedure, and special care must be taken to separate these two enzymes. The co-purification of the enolase isoenzymes from creatine kinase BB is detailed elsewhere (15).

Preparation of antisera. Antibodies were raised in Chinchilla bastard rabbits (IvACHB) by injecting 0.75 mg of the pure isoenzyme protein intradermally at several sites on the back. For the first injection we included 0.5 mL of complete Freud's adjuvant. The subsequent injections, at three-week intervals, included incomplete Freud's adjuvant. Seven days after the last injection, we collected serum from the animals. Most of them had developed antibodies after the second injection, as demonstrated by the double-diffusion test as well as by the inhibition of enolase activity in the photometric assay system (9).

Figure 1 shows the lack of cross reactivity between anti-αα-serum and γγ-enolase or between anti-γγ-serum and αα-enolase, although both antisera cross reacted with the hybrid form, αγ-enolase. There was also no cross reaction with purified creatine kinase BB, showing that it was not present as a contaminant.

Coupling of pure enolase isoenzymes to CNBr-activated Sepharose. We coupled 20 mg of pure γγ-enolase to CNBr-activated Sepharose as follows. Thoroughly wash 3 g of the dry Sepharose with 1 mmol/L

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1 Enolase isoenzymes αα, αγ, and γγ are designated enolase-1, -2, and -3, respectively, in some reports.
We antiserum NaHCO₃, per their ions. Their fraction 0.5 mol/L, 100 μg, by rotating the tubes overnight at room temperature. (Measurement of protein and of the inhibiting activity of the antibody solution before and after coating indicated that about 2 μg of antibody protein is adsorbed to the plastic wall of each tube.) Wash the coated tubes with several 2-mL portions of 0.1% bovine serum albumin solution to saturate unspecified binding sites, and store at 4°C. The same coating procedure can be used with polystyrene plastic beads, 0.4 mm diameter, instead of tubes.

**Solid-phase immunobiloluminescence assay for γγ-enolase.** The assay for γγ-enolase activity can be performed kinetically or as a fixed time assay. Table 1 summarizes the conditions of the reaction and the pipetting procedure.

For the kinetic assay, pre-incubate the sample and wash the tubes as detailed in Table 1. Then add 480 μL of the bioluminescence mixture (solutions 1–4 of step III), mix thoroughly, and initiate the reaction by adding 10 μL of the 2-phosphoglycerate solution. Measure the rate of increase in light emission. Add 10 μL of the ATP-standard solution to the sample to calibrate the assay system. An example of this type of assay is given in Figure 2.

For the fixed-time assay pre-incubate and wash, as above, then initiate the enolase–pyruvate kinase reaction by adding 450 μL of a mixture of 2-phosphoglycerate, pyruvate kinase, and ADP (solutions 2, 3, and 5 in Table 1). Incubate for a fixed interval (e.g., 1 h) at 25°C. After adding 40 μL of a solution of luciferin–luciferase (solution 4), measure the ATP accumulated during pre-incubation. Calibrate with 10 μL of the ATP-standard solution.

**Results and Discussion**

**Optimization of the Assay Conditions**

Figure 3 shows the time course of binding of γγ-enolase to the antibody coated on the tube. After 18 h at 25°C, all of the enzymatic activity was fixed to the tube. When coated tubes were incubated under these conditions, nearly all of the enzyme, up to a concentration of 200 ng of enolase per...

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**Table 1. Reaction Conditions for Determination of Enolase in the Present Assay**

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution</th>
<th>Final concn</th>
<th>Vol, μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Step I. Pre-incubate for 18 h at 25°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sample (if necessary, diluted with buffer)</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Buffer:</td>
<td></td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>Tris acetate, pH 7.75</td>
<td>100 mmol/L</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>2 mmol/L</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mg acetate</td>
<td>10 mmol/L</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bovine serum albumin</td>
<td>10 g/L</td>
<td></td>
</tr>
<tr>
<td>II.</td>
<td>Step II. Decant the sample and wash twice with 2.0-mL portions of buffer</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1. Buffer</td>
<td>as above</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>2. ADP</td>
<td>0.5 mmol/L</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>3. Pyruvate kinase</td>
<td>2 μL</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>4. ATP-monitoring reagent*</td>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>5. 2-Phosphoglycerate</td>
<td>0.9 mmol/L</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>6. ATP standard</td>
<td>variable</td>
<td>10</td>
</tr>
</tbody>
</table>

*ATP-monitoring reagent (LKB-Wallac) contains, per vial: firefly luciferase, α-luciferin, bovine serum albumin (50 mg), Mg acetate (0.5 mmol), inorganic phosphate (0.1 mmol), all to be dissolved in 5 mL of doubly distilled water.
The pure enzyme was incubated with antibodies for 2 h at 25 °C in a total volume of 0.3 mL. After 2 h we measured the enzyme activity with the kinetic bioluminescence assay, using the reaction sequence in Table 1. Up to 200 ng of γ-enolase per 0.3 mL we found no enzymatic activity in solution. For coated tubes we used the bioluminescence system to measure the activity in aliquots of the solution; we found no activity at the wall of the uncoated tubes.

The detection range of the assay is limited mainly by two factors: the upper detection limit is fixed by the amount of antibodies coating the tubes; the lower detection limit is influenced by contamination of reagents with ATP and by the type of antibodies used by coating. A high proportion of activity-inhibiting antibodies would raise the lower detection limit. It is therefore necessary to characterize the antibodies before and after coating. The lower detection limit of the fixed-time assay with a prolonged incubation was in the range of 5 × 10^-18 U, corresponding to about 0.1 pg (10^-18 mol) of enzyme protein. Thus, this assay is as sensitive as the enzyme immunoassay recently described by Kato et al. (8).

Measurement of Enolase Activities

To calculate the enzymatic activity bound to the specific antibody, one can either use a calibration curve with pure γ-enolase as a standard, or add a known amount of ATP to the assay system, using the following formula:

\[ \text{Activity, mU/L} = \frac{(\text{ATP}_\text{st} \times S_p \times DF) \times (J \times F)}{L} \]

where ATP_st is the concentration of ATP standard (in mmol/L), S_p is the rate of light emission per minute (from ATP formation via the enolase–pyruvate kinase reaction), J is the immediate increase in light emission after addition of ATP standard, DF is the dilution factor, needed to express the activity as mU/L, and F is the factor to correct for enolase activity inhibited by binding to the antibody.

Incubation of serum in tubes coated with anti-γ-enolase binds γ- and α-γ-enolase isoenzymes to the tube wall; incubation in tubes coated with anti-γ-enolase extracts the α- and γ-dimer. For specific detection of only one isoenzyme—for example, the γ-isoenzyme—we pre-incubate the sample first in anti-α-γ-coated tubes to remove α-isoenzymes activities, then we incubate the supernate in anti-γ-coated tubes. Analytical recovery experiments with pure α- or γ-enolase, respectively, show that the remaining activity is due to γ-enolase. By reversing the order of incubation, we can detect specifically the α-isoenzymes. Table 2 gives...
Table 2. Activities and Calculated Concentrations of Enolase Isoenzymes in Plasma

<table>
<thead>
<tr>
<th>Healthy subjects (n = 40)</th>
<th>( \gamma )-Enolase</th>
<th>( \alpha )-Enolase</th>
<th>( \alpha \alpha )-Enolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>mU/L</td>
<td>( \mu)g/L</td>
<td>mU/L</td>
<td>( \mu)g/L</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>165 ± 75</td>
<td>3.3 ± 1.5</td>
<td>300 ± 160</td>
</tr>
<tr>
<td>Range</td>
<td>25-350</td>
<td>0.5-7</td>
<td>60-700</td>
</tr>
</tbody>
</table>

Patients with small-cell lung cancer
1. 750 10 500 10 26 100 290
2. 1150 16 800 16 18 800 239
3. 1600 12 600 12 14 200 158
4. 1000 6 300 6 1800 20
5. 8000 160 50 1 15 000 167
6. 850 17 0 0 5200 58
7. 1400 28 2050 42 19 800 220

Patients with other malignant diseases (single cases)

Large-cell lung cancer
150 0.6 950 19 3300 37
Lymphoma
200 4 n.d. n.d. n.d.
Pancreas carcinoma
400 8 3800 76 0 0
Breast cancer
700 14 350 7 2200 24
Myeloid leukemia
600 12 100 2 35 000 389
Myeloid leukemia
750 15 200 4 2800 31
Thyroid carcinoma
700 14 2300 46 5800 64

values obtained by use of this procedure. The within-run coefficients of variation (\( n = 20 \) determinations) were 6% and 8% for the fixed-time and the kinetic assay of \( \gamma \)-enolase (activity: 100 mU/L), respectively.

This assay measures enzyme activities. To compare our results with data obtained with methods that measure enzyme protein (3, 4, 18), we first had to transform the activity values into enzyme concentrations. Because we do not know the specific activities of native enolase isoenzymes—i.e., in plasma—we used the specific activities of our purified enzymes (\( \alpha \alpha \)-enolase: 90 kU/g of protein; \( \gamma \)-enolase: 50 kU/g of protein). In general, the values for serum were greater than those for plasma, consistent with data in the literature (4) and suggesting that some of the serum enolases may be released from blood cells during clotting. Because thrombocytes, erythrocytes, and lymphocytes contain considerable amounts of the three enolase isoenzymes (4, 6), blood-cell lysis must be avoided during sampling. We therefore prefer to use plasma instead of serum.

Our calculated values (mean ± SD) for the concentrations of the three enolase isoenzymes in plasma of 40 ostensibly healthy subjects were 24 ± 10, 6.0 ± 3.2, and 3.3 ± 1.5 \( \mu\)g/L for \( \alpha \alpha \), \( \alpha \gamma \), and \( \gamma \gamma \)-enolase, respectively. Kato et al. (4) reported 20 ± 6, 4.1 ± 1.4, and 1.5 ± 0.4 \( \mu\)g/L.

In pilot studies with the solid-phase immunoluminescence assay we measured enolase isoenzymes in plasma of some patients with various malignant diseases. In all seven patients with small-cell lung cancer we found increased \( \gamma \gamma \)-enolase activities, two-to-20-fold the upper limit of normal. Some of these cases also had increased \( \alpha \alpha \)-isoenzyme activities in plasma. Again, the results are consistent with reported data (4). A patient with acute myeloid leukemia showed slightly increased \( \gamma \gamma \)-activities, but drastically increased \( \alpha \alpha \)-activities. Patients with solid tumors generally had normal \( \gamma \gamma \)-activities, but some had increased \( \alpha \alpha \)-activities. Further detailed studies are needed to elucidate the specificity of enolase isoenzymes for detection of malignant tumors.

The analytical advantages of this solid-phase immunoluminescence assay for enolase isoenzymes can be summarized as follows:
- It is highly sensitive, allowing separate determination of enolase isoenzymes in body fluids, even from healthy subjects. Measurements in cerebrospinal fluid as well as in tissue homogenates are possible.
- It is more specific than methods involving only specific antibodies for detection of the enzyme proteins, because measurement of enzymatic activity clearly increases the specificity of the assay.
- Use of the solid-phase binding to the specific antibodies avoids possible interferences within the bioluminescence detection system. Thus ATP or ATP-regenerating enzymes in plasma, such as adenylyl kinase, cannot interfere as in the luminescence assay systems described for creatine kinase (11).
- The coated tubes can be re-used. Acidifying the tubes after the assay by adding Tris-glycine buffer (pH 2.5) for 60 min to liberate the bound enolase, then washing twice with assay buffer containing 10 g of bovine serum albumin per liter, produced no loss in antibody-binding capacity. So far, we have used the same coated tubes in seven consecutive test procedures.
- Bioluminescence reagents and the equipment (the luminometer) are commercially available.
- Because of its high concentrations in brain, preparation of \( \gamma \gamma \)-enolase is relatively easy.

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References


