LABELING OF CREATINE PHOSPHOKINASE WITHOUT LOSS OF ENZYME ACTIVITY

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Summary: Creatine phosphokinase (CPK) loses enzyme activity after direct iodination by the chloramine-T, thallic trichloride and lactoperoxidase methods. Radioiodination of CPK without loss of enzyme activity can be achieved by Bolton-Hunter reagent which conjugates at the N-terminal or ε-lysyl NH_2 group of the protein.

Use of ^{125}I-labeled proteins and polypeptides for tissue distribution and metabolic studies is a legitimate approach, if the labeled products retain their full biological activity. The use of ^{125}I-labeled enzymes and hormones in radioimmunoassay may not necessitate strict preservation of molecular structure for biological action. Nevertheless, quantification will be more authentic when the tracer used is in the biologically active form.

The widely used chloramine-T method (1) is a simple iodination procedure to produce labeled proteins of high specific activity by a direct introduction of the isotope into the tyrosyl and histidyl residues of the peptide chain. However, such a procedure can alter the tertiary structure and impose iodination damage on the molecule and consequently, cause loss of biological activity as is the case with parathyroid hormone (2). Iodination by enzymic method (3, 4) reduces the damage due to exposure of the protein to oxidizing and reducing agents. However, the steric effect of relatively large radioiodine atoms on the molecular configuration remains. Recently, Bolton and Hunter (5) developed an acylating agent, 3-(*p*-hydroxyphenyl)-propionic acid N-hydroxysuccinimide ester, which can be iodinated by conventional chloramine-T technique.

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In an aqueous solution, the iodinated ester reacts with a protein or polypeptide by conjugation at the free amino groups (both N-terminal and ε-lysine). It has been reported that human growth hormone antiserum binds more avidly with the tracer produced by the conjugation labeling technique than with directly iodinated tracer (6).

In this communication, we are describing the successful iodination of creatine phosphokinase (CPK) with the iodinated ester. The labeled CPK is preserved with full enzyme activity. Iodination of CPK with other methods results without exception in a substantial loss of enzyme activity even with the necessary precautions taken to protect the active site of the enzyme molecule.

MATERIALS AND METHODS

Enzymes and chemicals: Creatine phosphokinase, CPK, (EC 2.7.3.2) prepared from rabbit muscle was purchased from Miles-Seravac (Pty) Ltd., Maidenhead, Berks, England (29 U/mg) and from Sigma Chemical Co., St. Louis, Mo. (115 U/mg). For each iodination, the freshly dissolved CPK solution in 0.01 M phosphate buffer-0.15 M saline (PBS), pH 7.5, was used. Lactoperoxidase (EC 1.11.1.7) purchased from Sigma was dissolved in equal volumes of acetate buffer, pH 5.7, and glycerol and stored at -20°C in concentration of 1 µg/µl. Chloramine-T (Cl-T) was purchased from Eastman Organic Chemicals, Rochester, N.Y., and only the freshly prepared solution was used. Thallic trichloride ($TlCl_3$) was purchased from K & K Laboratories, Plainview, N.Y., and stored at room temperature in 0.1 M concentration.

Isotopes: Carrier-free $Na^{125}I$ with a minimum specific radioactivity of 300 mCi/ml was purchased from Amersham/Searle Corp., Arlington Heights, Ill. ^{125}I-labeled Bolton-Hunter acylating reagent in benzene solution was a product of New England Nuclear, Boston, Mass.

Iodination procedures:

1. Chloramine-T method (1): In a reaction vial containing 25 µl of 0.5 M phosphate buffer, pH 7.5, the following reagents were added in the listed sequence: a. 1 mCi or 0.02 mCi (50 times dilution with equivalent concentration

of stable NaI) of $Na^{125}I$ in less than 2 µl. b. 10 µl of CPK solution (290-575 mU) in PBS. c. 2 or 20 µl of Cl-T solution in PBS (mg/ml). d. 1 or 10 µl of sodium meta-bisulfite, $Na_2S_2O_5$, solution in PBS (10 mg/ml). e. 50 µl of KI in PBS (4 mg/ml) containing 0.2% gelatin (PBS-G). In one experiment, 10 µl of 0.04 M creatine phosphate was added 2 min prior to the addition of Cl-T. In another experiment, 10 µl of 0.001 M *p*-chloromercuriphenyl-sulfonic acid (PCMPS) was added 5 min before Cl-T and then 10 µl of 0.02 M dithiothreitol (DTT) replaced $Na_2S_2O_5$. The oxidation time was 30 sec.

2. Thallic trichloride method (7): The basic procedure was identical to chloramine-T method except that 5 µl of 0.1 M $TlCl_3$ was used as oxidant instead of Cl-T. The reaction time was 5 min.

3. Lactoperoxidase method (8): In a reaction vial containing 25 µl of 0.5 M phosphate buffer, pH 7.5, added in sequence were 1 mCi of $Na^{125}I$, 10 µl of CPK (1.15 U) in PBS, 5 µl of lactoperoxidase solution and 5 µl of diluted hydrogen peroxide (10 µl of 30% H_2O_2 in 150 ml of H_2O). The reaction proceeded for 5 min. After the addition of the second 5 µl of diluted H_2O_2, the reaction continued for 5 min and was terminated by addition of 1.0 ml of PBS.

4. Conjugation method (5): An aliquot of the benzene solution of the ^{125}I-labeled ester, 300-400 µCi, was transferred to a small reaction vial. The solvent benzene was evaporated in a closed hood with strong air-drifting for 5-10 min. The vial was cooled in ice for 10 min before 100 µl of CPK solution (290 mU in 0.1 M borate buffer, pH 8.5) was added directly to the vial. The reaction vial was rotated in ice for a period of 15, 30 or 45 min. To terminate the reaction, 0.5 ml of 0.2 M glycine solution in borate buffer was added to the vial followed by 5 min rotation.

Purification: Reaction mixture of the lactoperoxidase procedure was fractionated in a Sephadex G-100 column (2 x 60 cm), void volume of 45 ml, and eluted with PBS-G in 2.5 ml fractions. The reaction mixture by other procedures was fractionated with disposable Sephadex G-75 column (0.9 x 20 cm), void volume of 4 ml, and eluted with PBS-G in 0.4 ml fractions. The gel column was pre-

equilibrated with PBS-G. Before fractionation of the iodinated enzyme, a preparation of CPK containing the same amount of enzyme was fractionated in the same column. This provided a control for determination of enzyme activity of the uniodinated CPK.

Enzyme activity assay: CPK activity was determined by a spectrophotometric procedure of Rosalki (9).

Calculations: The incorporation of ^{125}I into the CPK was calculated by the percentage of radioactivity monitored in the whole protein peak after gel filtration. The enzyme activity in all fractions of the protein peak was integrated. The percentage of iodinated CPK activity was based on that of each individual control CPK preparation.

RESULTS

The incorporated radioactivity and the retained enzyme activity of ^{125}I-CPK labeled by different procedures are listed in Table 1. By the conventional chloramine-T method, CPK can be easily iodinated with 57.5% efficiency. Although the average radioactivity is 115 μCi/μg, or 4.3 atoms of radioiodine per molecule of CPK (assumed to be pure at 115 U/mg and having mol. wt. 81,000), the peak fraction had specific radioactivity of 550 μCi/μg, 94% of which could be precipitated by 30% CCl_3COOH. The iodinated CPK, however, preserved only 15% of the total enzyme activity. When the isotopic iodide was diluted with stable iodide before labeling, the product incorporated an average of less than 0.45 atoms of ^{125}I per molecule and retained only 25% of the control enzyme activity. When the diluted isotope reacted with CPK using one tenth the amount of Cl-T and $Na_2S_2O_5$, the product contained an average of less than 0.12 atoms of ^{125}I per molecule but still lost 30% of its enzyme activity. Neither the enzyme substrate, creatine phosphate, nor PCMPS, a thiol group protector, could protect the enzyme from the noxious effect of iodination on its biological activity. Thallic trichloride, a milder oxidant than Cl-T, did improve the preservation of enzyme activity somewhat. In iodination of CPK with lactoperoxidase to avoid the use of strong oxidizing and reducing agents, 71% of

TABLE 1

RADIOACTIVITY AND ENZYME ACTIVITY OF ^{125}I-CREATINE PHOSPHOKINASE LABELED BY DIFFERENT PROCEDURES

Iodination Procedure	Radioactivity μCi/μg	Enzyme Activity %
Chloramine-T methods:		
Conventional	115	15
50x dilution of $Na^{125}I$	12	25
10x dilution of Cl-T and $Na_2S_2O_5$	3	70
Creatine phosphate protection	30	25
PCMPS protection	124	28.3
PCMPS protection and 10x dilution of Cl-T and $Na_2S_2O_5$	4.2	55
Thallic trichloride method:	14.6	64
Lactoperoxidase method:	6.5	71
Conjugation method, 15 min:	11	101
30 min:	12.4	111
45 min:	13.6	102

the activity of ^{125}I-CPK product was retained. However, the preservation of some enzyme activity of ^{125}I-CPK by $TlCl_3$ or enzymic iodination might be attributed to the light iodination of the CPK molecules. Full enzyme activity was retained by CPK labeled with ^{125}I-ester for 15 min. Prolonged conjugation time increased the radioactivity slightly and fully preserved the enzyme activity. The chromatogram of ^{125}I-acylated CPK is shown in Fig. 1. It is interesting to note that the peak enzyme activity of ^{125}I-CPK appeared one fraction earlier than the control CPK. This indicates that the molecular weight of the labeled CPK is clearly higher than the native enzyme. However, the changes of enzyme activity in the different fractions are coincidental with the changes

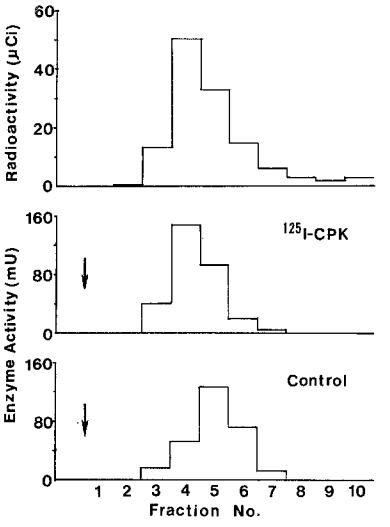

Figure 1. Chromatography of control CPK and ^{125}I-acylated CPK (30 min reaction). The void volume is indicated by ↓. Each fraction has 0.4 ml.

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of radioactivity. The specific radioactivity of fractions after peak increases progressively. This is due to contamination by other isotopic products, most likely glycine conjugated with the labeled acylating reagent. In fraction 4, which has the highest radioactivity and enzyme activity, the specific radioactivity is 0.337 μCi/mU or 9.8 μCi/μg, which is lower than the overall specific radioactivity of 12.4 μCi/μg (Table 1).

DISCUSSION

Enzymes sensitive to oxidizing or reducing agents lose biological activity when iodinated by the conventional chloramine-T method (10-12). Thallic trichloride or lactoperoxidase iodination may alleviate the damage somewhat.

CPK is a relatively stable protein and has two essential thiols which maintain the geometry of the active site configuration in the molecule (13). It loses enzyme activity whenever direct iodination is carried out by any one of the three conventional procedures. Acylation at the NH_2 group by Bolton-Hunter reagent has produced radioiodination without any loss of enzyme activity.

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