Labeled DIP-thrombin

It was demonstrated by GLADNER AND LAKI[1] and also by MILLER and VAN VUNAKIS[2] that thrombin similarly to trypsin[3], and chymotrypsin[4] reacted with di*iso*propyl-phosphorofluoridate (DFP) to give inactive di*iso*propylphosphoryl-thrombin (DIP-thrombin). In order to find the amount of thrombin that reacted with 1 mole of DFP, the inactivation reaction was carried out with ^{32}P-labeled DFP. Twenty thousand NIH units of Parke-Davis thrombin (topical) in 10 ml 0.5 *M* phosphate buffer pH 8.0, were inactivated by incubation at 25° for 20–60 min with 13–16 mg of ^{32}P-labeled DFP (obtained from Technical Operations, Arlington, Mass.) containing a total activity of $2–3 \cdot 10^8$ c.p.m. The reaction mixture was then dialyzed against 0.05 *M* phosphate buffer, pH 7.0, at 4° for 24 h, and the contents of the dialyzing bag were fractionated on an ion exchange column using a modification of the method of RASMUSSEN[5]. After the unbound DF^{32}P and contaminating proteins had separated from the column, a sharp radioactive peak emerged at exactly the same place and in the same volume as native thrombin in similar experiments. (From the solution containing the radioactive peak, thrombin can be reactivated by nucleophilic reagents such as hydroxamic acid[1].) This solution of thrombin contained about 0.6 mg protein/ml. When this solution was dialyzed against 0.05 *M* phosphate buffer pH 7.0, in the cold, about 80% of the radioactive material precipitated, similarly to native thrombin where 80% of the thrombin activity precipitated. The precipitate was centrifuged in a preparative ultracentrifuge to a small pellet. The pellets containing 8–9 mg protein were dissolved in 1–2 ml 0.5 *M* phosphate buffer, pH 8.0, and used for chemical, physical, and physiological experiments. Radioactivity in these experiments was determined in the dried samples in a gas flow counter with a micromil window. The ^{32}P bound to thrombin amounted to approximately 1% of the total radioactivity of the DF^{32}P used. On the assumption that this thrombin preparation is pure, and that the combination of DFP with thrombin is 1:1 (as in the case of trypsin[3] and chymotrypsin[4]) a tentative mol. wt. of 13,700 for thrombin can be calculated (Table I). This figure is in the same order of magnitude as the minimum mol. wt. of 13,000 ± 500 calculated from amino acid analysis[6].

TABLE I

DFP *mg*	*Total protein* *mg*	*Total P bound* *µg*	*Molecular weight*
13.1	10.3	37.7	9,900
16.4	10.6	25.0	13,100
15.0	11.3	26.5	13,200
14.0	11.0	20.0	17,000
14.0	2.26	4.5	15,500

The first column gives the amount of DF^{32}P used to inactivate separate lots of 20,000 NIH units of thrombin. The second column gives the amount of protein (DI^{32}P-thrombin) recovered in the radioactive protein peak. The third column is the amount of total P (determined from radioactivity) bound to the protein.

The fate of the inactive, but labeled thrombin (equivalent to 10,000 NIH thrombin units in 0.5 ml) was determined after intravenous injection into rabbits weighing 1.5 kg. The residual radioactivity from the plasma gave a curve which was curvilinear when plotted on semi-logarithmic paper with over 80% of the radioactivity being removed by the end of 2 h. Tissue radioactivity was found greatest in the lungs. A more detailed account of these investigations will be presented elsewhere.

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[1] J. A. GLADNER AND K. LAKI, *Arch. Biochem. Biophys.*, 62 (1956) 501.
[2] K. D. MILLER AND H. VAN VUNAKIS, *J. Biol. Chem.*, 223 (1956) 227.
[3] E. F. JANSEN AND A. K. BALLS, *J. Biol. Chem.*, 194 (1952) 721.
[4] E. F. JANSEN, M. D. FELLOWS-NUTTING, R. JANG, AND A. K. BALLS, *J. Biol. Chem.*, 179 (1948) 189.
[5] P. S. RASMUSSEN, *Biochim. Biophys. Acta*, 170 (1955) 154.
[6] D. R. KOMINZ, unpublished data.

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