High Specific Activity Iodination and Displacement Radioimmunoassay of Fructose-1,6-diphosphatase[1] (35601)

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Hormones such as insulin (1, 2), glucagon (3, 4), adrenocorticotropic hormone (5), and growth hormone (6) can be quantitatively assayed by displacement radioimmunology. The sensitivity and specificity of the assay is usually satisfactory to assay millimicrogram quantities of the hormones directly in their biologic environment. The method is based on the decreased binding of the radioiodine-labeled protein to its antibody, caused by increasing quantities of the unlabeled protein added as unknown or standard. To assay small amounts of protein by this method, a labeled form with high specific activity is required and is usually obtained by iodination with 125 or 131 iodine isotopes (7, 8).

It was of interest to determine whether enzymes of much higher molecular weight, composed of subunits and possessing allosteric properties, could also be determined quantitatively by radioimmunoassay. Fructose-1,6-diphosphatase from rabbit liver was considered a suitable model enzyme (9). The present paper describes the high specific activity iodination and the development of a radioimmunological assay for FDPase which offers a new method for the quantitative estimation of the enzyme on the basis of its protein characteristics.

Materials and Methods. Fructose-1,6-diphosphatase tetrasodium salt, triphosphopyridine nucleotide, phosphohexose isomerase, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Company. Carrier-free iodine 125 and 131 isotopes in small plastic vials were obtained from Iso-Serve (Division of Cambridge Nuclear Corp.). Chloramine-T was purchased from Eastman Kodak Organic Chemicals; sodium metabisulfite from Allied Chemicals. Frozen rabbit livers were obtained from Pel-Freez Biologicals; Inc. (Rogers, Ark.). All other chemicals, used for buffers, etc., were analytical reagent grade.

Fructose-1,6-diphosphatase (FDPase) was isolated from rabbit livers as described (10): from 600 g of frozen rabbit livers, 6–8 mg of enzyme were obtained. The final specific activity was 120–130 units/mg of protein. The enzyme was crystallized and checked for impurities by polyacrylamide gel electrophoresis, as described (11). At pH 8.3, 7% acrylamide concentration, a single protein band was obtained after staining with amido black. The biologic activity of FDPase was determined with fructose-1,6-diphosphatase as substrate in 0.1 *M* glycine buffer, pH 9.4, as described by Pontremoli *et al.* (10). Protein was determined by the method of Lowry *et al.* (12) with bovine serum albumin as standard. For the purified enzyme, the protein was estimated using the molar extinction coefficient of Pontremoli *et al.* (10).

Fructose-1,6-diphosphatase antiserum was obtained from guinea pigs after four weekly consecutive injections of 1 mg of enzyme suspended in 0.2 ml of Freund's adjuvant. Five animals were injected in the foot pads, and blood was taken by cardiac puncture. The serum was stored in small portions at −30°.

Radioactivity was determined by using a Model 1085 automatic gamma well counter or a table monitor, both from Nuclear-Chicago.

Two different procedures previously used for radioiodination of hormones have been applied for the iodination of FDPase. The iodine 131 isotope employed as "carrier-free" contained about 20–40% radioiodine. For

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TABLE I. Iodination with ^{131}I of Fructose-1,6-diphosphatase Using Iodine Monochloride. 5 μl of radioiodide = 2.5 mCi were used for the iodination. The reaction time was 5 min.

No.	FDPase (μg)	Moles of ICl/ moles of FDPase	Yield of iodination (%)	Sp act (mCi/mg of protein)
1	30	1	0	0
2	30	10	0	0
3	30	50	2	1.6
4	80	10	6	1.9
5	300	1	4	0.3
6	310	4	20	1.6
7	310	10	29	2.4
8	300	50	32	2.6
9[a]	300	10	24	8

[a] 20 μg of radioiodine = 10 mCi used for iodination.

comparative purposes, the same shipment of radioiodine was used for a given series of experiments.

Iodination using iodine monochloride (ICl). Iodine monochloride was prepared as described by Izzo *et al.* (8). The 0.02 *M* stock solution in 0.04 *M* KCl was kept in the refrigerator and diluted with 0.02 *M* NaCl solution to the desired concentration. The radioiodide was preincubated to destroy the hydrogen peroxide formed by radiation: 5–10 μl of radioactive iodide were incubated in 50 μl of 0.4 *M* borate buffer, pH 8.4. To this was added 50 μl of 0.5 *M* sodium sulfite, and the mixture was incubated in a 70° water bath for 2 hr. During this procedure, about 20% of the radioactive iodide was lost by evaporation. After cooling, 30–300 μg of FDPase in 50 μl of 0.4 *M* borate buffer (pH 8.4), and 20–50 μl of iodine monochloride solution in 0.02 *M* sodium chloride were added. After 2–5 min, the unreacted iodide was separated by passing the solution over a Sephadex G-25 column (1 × 14 cm, 3 g Sephadex). The percentage yield of iodination was calculated from the ratio of radioactivity in the sample. The iodinated FDPase was stored in an albumin solution (10 mg/ml) and kept frozen at —30°.

High specific activity iodination using chloramine-T. Iodinations with chloramine-T as oxidant were done as described (7). 20–50 μg of FDPase in 0.5 *M* phosphate buffer (pH 7.6) and the radioactive iodide (1–10 μl; 1 μl = 0.5 mCi) were added to a conical tube and carefully mixed. Iodination was started by adding, with mixing, 25 μl of freshly prepared chloramine-T solution (usually 4.2 mg/ml in 0.5 *M* phosphate buffer) and terminated with 100 μl of freshly prepared sodium metabisulfite solution (5 mg/ml). The unreacted iodide was separated from the enzyme by passing the mixture over the Sephadex G-25 column.

Results. High specific activity iodination using iodine monochloride. Before use, it was necessary to preincubate the radioactive iodine isotope with sodium sulfite to destroy hydrogen peroxide present in the commercial radioiodide solution. The FDPase concentrations and the amount of iodine chloride used for the iodination were varied. The results of these experiments are shown in Table I. At protein concentrations from 20 to 80 μg, the yields of radioiodinated enzyme were low. At high concentration of radioiodine, the specific activity of the radioiodinated enzyme did not exceed 8 mCi/mg of protein.

High specific activity iodination using chloramine-T as an oxidant. Since the commercial radioactive iodide is used without further purification in this procedure, an excess of the oxidant chloramine-T is necessary to prevent re-reduction of iodine by hydrogen peroxide. The amount of enzyme was kept at 20–50 μg since it was desirable to obtain a high specific activity of the enzyme without using excessive amounts of radioactive iodine. Specific activity of radioiodinated FDPase increased with increasing concentra-

TABLE II. Iodination with ^{131}I of Fructose-1,6-diphosphatase Using Chloramine-T.

No.	Amount of enzyme (μg)	Cl-T used (moles)	Incubation time (sec)	Yield of iodinat. (%)	Sp act (mCi/mg of protein)	Biological activity (%)
1	26	3×10^{-7}	7	28	26	65
2	26	3×10^{-7}	20	26	25	35
3	26	3×10^{-7}	60	34	32	5
4	20	3×10^{-7}	300	69	87	0
5	30	3×10^{-8}	60	0	0	—
6	40	10^{-7}	60	5	3	32
7	40	10^{-7}	300	8	5	3
8[a]	20	3×10^{-7}	60	1	1	85
9[b]	20	3×10^{-7}	60	6	5	62
10[c]	30	3×10^{-7}	60	42	35	28

[a] Dithiothreitol present (moles): 5×10^{-7}; [b] 3×10^{-7}; [c] 10^{-7}.

tions of chloramine-T and increasing exposure times (Table II); however, these conditions also caused progressive lack of biologic activity. Reducing agents like mercaptoethanol or dithiothreitol in concentrations of 10^{-7} *M* protected the enzyme against inactivation by chloramine-T, but the yield of iodination dropped prohibitively. Metal complexing agents like EDTA did not increase yields. As shown in Table II, optimal specific activity, with minimal loss in biologic activity, was achieved when incubation times were reduced to 7 sec.

Displacement radioimmunoassay of fructose-1,6-diphosphatase. In the displacement assay it is necessary to separate free antigen from antigen bound to the antibody. This was achieved by preferential precipitation of the antigen–antibody complex with sodium sulfate, a method previously employed in this laboraory for the immunoassay of insulin (2, 13), glucagon (14), and human growth hormone (15). For best results in the immunoassay, nonspecific precipitation of free radioiodinated enzyme should be minimal. Figure 1 shows the sodium sulfate precipitation curves of native and ^{131}I-FDPase, radioiodinated by the chloramine-T method in absence of antiserum. At pH 7.0 and 8.5, identical precipitation behavior was observed. Curve 1 shows that the native enzyme, at the dilute concentration required for immunoassay, remained soluble in the absence of antibody even at a high ratio (5:1) of 25% sodium sulfate to incubation volume. In contrast, enzyme iodinated by chloramine-T for 300 sec and with a specific activity of 87 mCi/mg was less soluble (Curve 5). Howev-

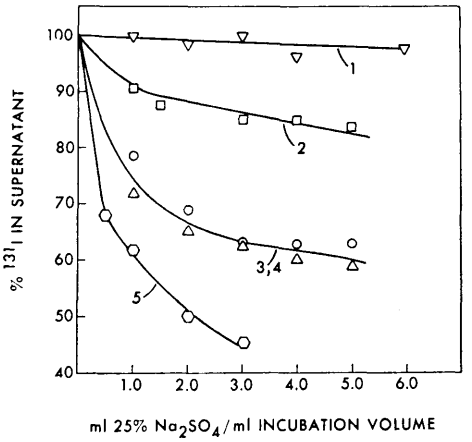

FIG. 1. Precipitation of 131 iodinated FDPase by increasing concentrations of 25% sodium sulfate solution: The incubation volume was 1.0 ml in 0.2 *M* glycine buffer (pH 7.6) containing 10 mg of bovine serum albumin and 5 mg of human gamma globulin. After the addition of the sodium sulfate, the sample was incubated at 25° for 30 min and centrifuged at 5000 rpm at room temperature. An aliquot of the supernatant was counted. The enzyme was iodinated by the chloramine-T method. (1, ▽) native FDPase; (2, □) iodinated for 7 sec; (3, ○) iodinated for 30 sec; (4, △) iodinated for 1 min; and (5, ⬡) iodinated for 5 min.

er, iodoenzyme of adequate specific activity prepared at shorter incubation time, *e.g.*, 7 sec, permitted a good yield of radioiodination and had a solubility more closely resembling the native enzyme. The ratio of 25% sodium sulfate to incubation volume of (2.4), which was used for the immunoassay, caused about 20% of the labeled enzyme to precipitate nonspecifically.

Enzyme iodinated by the ICl method at a level of 10 and 50 moles of ICl/mole of FDPase also exhibited decreased solubility of about the same magnitude as after 60-sec chloramine-T treatment. Samples prepared at 1 mole of ICl/mole of enzyme had better solubility; however, the specific activity was only 0.3 mCi/mg of protein (Table II).

FDPase, iodinated with 131 iodine by the chloramine-T method at different incubation times was used as a label in the immunologic determination of native FDPase. The method was similar in principle to that previously described for insulin (2, 13), but differed in actual quantities of reagents. Different amounts of native FDPase were incubated with antiserum diluted 1:5000 in 0.6 ml of 0.2 *M* glycine buffer (pH 7.6) containing 1% albumin for 16 hr at 4°. Afterwards, 0.3 ml of a solution containing 1% albumin, 5 mg/ml of gamma globulin as carrier protein, and about 0.01 mμg of ^{131}I-FDPase in 0.2 *M* glycine buffer (pH 7.6) were added and the tubes were incubated at 25° for 15 min. After centrifugation an aliquot of the supernatant was counted.

In Fig. 2, the amount of 131 iodine displaced into the supernatant is plotted against the logarithm of FDPase concentration. These data show that the standard curve using iodoenzyme prepared at 7-sec exposure to chloramine-T was significantly more sensitive than the more inactivated and denatured preparation exposed for 60 sec. When FDPase, radioiodinated by ICl, was used as a label in identical experiments, the standard curve was only as sensitive as Curve 2 in Fig. 2.

The minimum sensitivity of the assay was about 0.01 enzyme units, which is equal to approximately 0.1 μg of FDPase/sample.

When FDPase concentations were determined in crude 100,000*g* supernatant of rabbit liver homogenate by the biological and radioimmunological assay, the same enzyme level was found by both procedures (16). No immunological activity was found in supernatants of rabbit heart, spleen, and brain, which contained little or no FDPase activity (16).

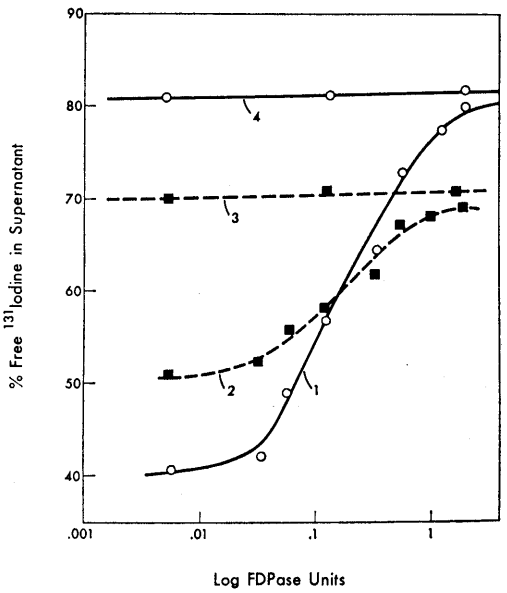

FIG. 2. Immuno-displacement of 131 radioiodinated FDPase by increasing concentrations of native enzyme. Two samples of FDPase, radioiodinated using chloramine-T (procedure II) at different incubation times are compared. The values shown represent the mean duplicates: (1 and 4, O, O) 7-sec incubation time for radioiodination; (2 and 3, ■, ■) 60-sec incubation time for radioiodination; and (3 and 4) represent controls in absence of antiserum.

Discussion. The use of iodine monochloride for radioiodination has the advantage of not exposing fructose-1,6-diphosphatase to such strong oxidants as chloramine-T. However, there are also a number of disadvantages: (i) Nonradioactive carrier iodine is incorporated into the protein. (ii) Large amounts of protein and radioiodine are necessary to give a specific activity iodination of 5–10 mCi/mg of enzyme. (iii) The radioiodine has to be pretreated to destroy the hydrogen peroxide. This requires, in addition to extra time and large amounts of radioiodine, safety precautions during

air oxidation of the sulfate. (iv) The yield of iodination is still comparatively low, and consequently a low specific activity of the protein is obtained. However, for procedures where a low specific activity of 2–5 mCi/mg of enzyme is sufficient, the iodine monochloride method may be useful.

The iodination procedure using chloramine-T does not require the addition of nonradioactive iodine to the reaction mixture. Consequently, high specific iodination is possible. Another advantage is that amounts as small as 2–3 μg of protein can be iodinated, resulting in specific activities of 25–500 mCi/mg of protein (7). A disadvantage of the procedure is that an enzyme molecule, sensitive to the oxidative environment, is much less soluble after treatment with chloramine-T, as demonstrated by the precipitation curve obtained. Reduction of the incubation time to 7 sec, however, yielded a good solubility of the enzyme while maintaining high specific activity.

Since the radioiodinated enzyme will be used as a tracer for the radioimmunoassay, the structural differences between the native and radioiodinated enzyme should be as small as possible. It should be emphasized, however, that one advantage of the radioimmunodisplacement assay is that exact identity of native and radioiodinated antigen is not required; reliability depends, instead, on similarity of unlabeled standard and unknown sample to displace the radioiodinated label from the antibody. The sensitivity of the radioimmunological displacement assay for the determination of FDPase was approximately that of the biological activity test, about 0.01 unit, or about 0.1 μg of enzyme. Even increased sensitivity, however, can be anticipated with an antiserum of higher titer (5).

The specificity of the immunoassay was indicated by the observation that FDPase could be estimated quantitatively in crude 100,000*g* supernatant of the rabbit liver, but was not detected in supernatants of rabbit heart, spleen, or brain. The assay, when compared to the biological activity test, is based on a completely different principle and should be useful for the investigation of structural differences or changes of enzymes in various species and tissues.

Summary. Displacement radioimmunoassay of fructose-1,6-diphosphatase is described, capable of measuring quantitatively 100 mμg of enzyme in biological fluids. The preparation of radioiodinated FDPase with high specific activity, a necessary prerequisite for the radioimmunoassay, was complicated by the lability of the enzyme and was, therefore, investigated in detail. Low specific activities of radioiodination in the range of 1–8 mCi/mg of protein were obtained by the ICl method. Using chloramine-T as an oxidant resulted in radioiodinated FDPase with specific activities in the range of 20–80 mCi/mg of protein. However, the enzyme proved to be sensitive against exposure to chloramine-T; best yields were obtained at short reaction times of less than 10 sec.

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