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Effect of ^{131}I -Labelling on Various Properties of Nonmitochondrial Aspartate Transaminase from Pig Heart

By S. MASSARRAT¹⁾

Medizinische Poliklinik, Philipps-University Marburg/Lahn, Germany

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Aspartate transaminase from the soluble fraction of pig heart cytoplasm (nonmitochondrial aspartate transaminase) was labelled with ^{131}I . The enzyme could be labelled up to 0.35 iodine atoms without loss of activity; with 2—6 atoms of iodine a loss of 60—85% activity was observed. With increasing iodination (more than 1.2 atoms per molecule) some change of the MICHAELIS constant from 0.28 ± 0.07 mM (normal value) to 0.14 ± 0.06 mM for 2-oxoglutarate, and from 8.6 ± 1.1 mM (normal) to 21 ± 8.7 mM L-aspartate appeared. The immune inhibition of the enzymes, the capacity of nonmitochondrial aspartate transaminase for binding antibody, and the electrophoretic mobility of the iodinated enzymes and their disappearance from the intravascular space after injection in the piglets remained almost unaffected.

Aspartat-Transaminase aus löslicher Fraktion des Schweineherzens wurde mit ^{131}I markiert. Das Enzymprotein erlitt bis zu 0,5 Atom Jod/Molekül keinen Aktivitätsverlust. Bei einer Zahl von 2—6 Atomen Jod pro Molekül wurde ein Verlust der Enzymaktivität von 60—85% beobachtet. Mit zunehmender Jodierung (mehr als 1,2 Atome Jod/Molekül) wurde eine Änderung der MICHAELIS-Konstante von $0,28 \pm 0,07$ mM (Normalwert) auf $0,14 \pm 0,06$ mM für 2-Oxoglutarat und von $8,6 \pm 1,1$ mM (Normalwert) auf $21 \pm 8,7$ mM für L-Aspartat festgestellt. Die immunologische Hemmbarkeit, die Kapazität für die Antikörperbindung, die elektrophoretische Wanderungsgeschwindigkeit sowie die Schwundrate des unterschiedlich jodierten Enzymproteins aus dem Intravasalraum nach Injektion bei Ferkeln blieben nahezu unbeeinflusst.

Several studies exist on the iodine labelling of the enzymes pepsin (1), α -chymotrypsin (2, 3, 4), ribonuclease (5, 6, 7), carboxypeptidase (8), lactate dehydrogenase (9, 10, 11), fructose diphosphate aldolase (12, 13), and glyceraldehyde 3-phosphate dehydrogenase (14). Much attention has been given to the alteration of the enzyme proteins by iodination. In previous work we have studied the immunologic behavior of nonmitochondrial aspartate transaminase²⁾ (15, 16—19), as these were of interest for the production site and the half-life of this clinically important serum enzyme. In this paper we have studied the extent to which the properties of non-mitochondrial aspartate transaminase are altered by iodination.

Loss of enzyme activity, alterations of the MICHAELIS constant, the immunological properties, and the electrophoretic mobility and biological behavior were examined after different degrees of iodination.

Materials and Methods

Enzymes, substrates, and coenzymes were obtained from Boehringer/Mannheim, Germany.

Aspartate transaminase from the soluble fraction of the cytoplasm was isolated from highly purified aspartate transaminase (pig heart, Boehringer/Mannheim) in a procedure similar to the method described briefly for the separation of lactate dehydro-

genase isoenzymes (20). This includes further purification of aspartate transaminase on DEAE-Sephadex (A-50, medium coarse) after dialysis with phosphate buffer 0.008M, pH 7 overnight. The enzyme was eluted stepwise with a 0.05M phosphate/0.1M NaCl buffer and recovered in the first peak with phosphate buffer (pH 6, 0.05M). This enzyme was used for ultracentrifugal analysis and immunization.

Ultracentrifugal analysis was carried out in a Beckman analytical ultracentrifuge Model E, equipped with schlieren optics. The experiment, in which the sedimentation constant of the enzyme protein was studied at two concentrations, was performed at a rotor speed of 59780 rpm. at 20°.

Iodination procedure and assay of iodine atoms per molecule

After dialyzing overnight with phosphate buffer 0.008M, pH 7 the enzyme proteins were labelled with ^{131}I as described by FRANCIS and coworkers (21), using a solution of KI 0.002M plus free iodine 264 mg/l. The protein solutions containing 0.9 to 5 mg protein/ml were made alkaline with ammonia. Usually 0.035 ml ammonia 14.7N was added to 5 ml of solution to make a final pH of 8.5—8.9. In agreement with the experiments of SHEID and coworkers (22), no substantial loss of enzyme activity was found in the pH range between 7.5 and 8.5; iodination of the enzyme is tolerated well in this range. Carrier-free Na^{131}I dissolved in NaOH 0.1N, obtained from Hoechst (Germany), was added to the I-KI solution, followed by HCl 0.1N until the solution turned light yellow. This solution was added to the protein solution slowly drop by drop with constant stirring. The pH of the solution was generally not below 7.2 after this procedure.

The iodinated protein solution was first diluted 1:500 or 1:1000 (depending on the enzyme activity) with phosphate buffer 0.1M, pH 7.5. The diluted solution was mixed with human serum (0.1 ml serum per 1 ml diluted solution), then precipitated with 10% trichloroacetic acid; the precipitate was dissolved in 0.5 to 1 ml 0.2N NaOH and precipitated again with 10% trichloroacetic acid. This procedure was repeated at least three times until the radioactivity in the precipitate remained unchanged. The percentage of protein-bound iodine was calculated from the ratio of the radioactivity of the last precipitate to that of the solution

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²⁾ *Enzymes*: Aspartate transaminase = L-aspartate: 2-oxoglutarate aminotransferase (EC 2.6.1.1); Malate dehydrogenase = L-malate: NAD oxidoreductase (EC 1.1.1.37); Glutamate dehydrogenase = L-glutamate: NAD oxidoreductase (deaminating) (EC 1.4.1.2).

before precipitation. In the control study 0.1 ml of the iodinated protein solution was mixed with 0.9 ml of human serum as a carrier protein, and dialyzed in Visking tube against phosphate buffer 0.1M pH 7.5 for 48 to 72 hrs, with repeated changes of buffer until no more radioactivity could be detected in the dialysate. The percentage of the protein-bound radioactivity was compared with that obtained by precipitation with trichloroacetic acid. The protein measurement was carried out according to LOWRY (23).

For the calculation of the number of iodine atoms per molecule a molecular weight of 78600 was assumed (24).

The radioactivity was measured in the γ -scintillation counter (Bohrlochkristall Friesseke & Höpfner, Erlangen, Germany). The specific radioactivity was 0.2–40 $\mu\text{Ci}/\text{mg}$ protein. The enzyme activity of the labelled protein was measured immediately following iodination in the 1:500 or the 1:1000 solution, and the iodinated aspartate transaminase separated on DEAE-Sephadex. The free iodine was removed in phosphate buffer having the lowest molarity (0.008M, pH 7). The proteins were eluted from Sephadex in the manner described above. The labelled enzyme from the first peak was collected and concentrated for the electrophoretic and biological experiments.

Measurement of enzyme activity

The enzyme activity was measured spectrophotometrically at 366 nm and 25°.

The reagents were as follows:

NADH 0.3 mM, 2-oxoglutarate 10 mM, aspartate 50 mM, phosphate buffer 0.1M, pH 7.5 (containing EDTA 6 mM), malate dehydrogenase roughly 370 mU/ml per test, total volume 1.0 ml, $d = 1$ cm.

The MICHAELIS constants were determined according to LINEWEAVER-BURK. Alteration of the MICHAELIS constant was estimated for two substrates, 2-oxoglutarate, and L-aspartate, by keeping the level of one constant, as described above.

The concentration of 2-oxoglutarate was measured enzymatically with glutamate dehydrogenase on the basis of the consumption of NADH. The following reagents were used:

NADH 0.72 mM, $\text{CH}_3\text{COONH}_4$ 20 mM, glutamate dehydrogenase 0.1 mg per test, triethanolamine buffer 0.05M, pH 7.5 (containing EDTA 6 mM). The concentrations of L-aspartate were calculated according to the data of the producer.

Immunologic experiments

The antibodies were obtained from rabbits by immunization with the enzymes in the method previously described (17). Immunoelectrophoresis was carried out according to GRABAR (25) in the method modified after MARTIN and coworkers (26). The immunological properties of the labeled proteins were determined by the inhibition of enzyme activity by specific rabbit antibodies (17) and by the precipitation method. 0.015 ml antiserum was incubated for 10 min. with 18 mU of native enzyme, or with a constant amount of iodinated enzyme corresponding to the 18 mU of native enzyme protein. The activity of the iodinated enzyme was measured before and after adding the antibody; then the percentage of immune inhibition was calculated. Immunological measurement of the enzyme protein was carried out using the precipitation method according to MANCINI's radial diffusion in agar gel (27).

Gel electrophoresis

Starch gel according to SMITHIES (28) and polyacrylamide gel according to RAYMOND and coworkers (29), as modified by ZWISLER (30), were used in the electrophoretic studies of the enzyme protein. For its identification amido black or specific aspartate transaminase stain was used.

Staining for aspartate transaminase

The solution used for incubation was selected according to DECKER and coworkers (31); it contained 12 mM 2-oxoglutarate, 40 mM L-aspartate, and 2.0 g/l of 6-benamido-4-methoxytolui-

din diazonium chloride (Alliance Chemical Corporation, New Jersey, USA) in 0.1M phosphate buffer pH 7.5. L-aspartate was omitted in the control solution. Immediately following addition of the diazonium salt the gels were incubated at 37° for about 20 min. Afterward they were washed with distilled water in order to remove the excess stain.

Biological animal experiments

Before the experiment was started, female piglets (Göttinger Zwergschweine, Institut für Tierzucht und Haustiergenetik Göttingen, Germany) with a body weight of 5–8 kg were given KI in the drinking water for the purpose of blocking the thyroid glands.

2 to 9 days after the iodination 0.5–1 mg of labeled protein with 5.7 iodine atoms per molecule and 72% loss of enzyme activity was mixed with 5 mg of unlabeled protein and injected into one piglet subcutaneously and into another intravenously. Blood was taken from the tail vein over a period of 1–2 days under general sedation with pentobarbital. The enzyme activity and protein-bound radioactivity were measured in the sera.

Results

Enzyme purity

The ultracentrifugal study shown in Figure 1 reveals a single peak of nonmitochondrial aspartate transaminase with a sedimentation constant of 4.86 S. The specific activity was 180 IU per mg protein.

In immunoelectrophoresis a single precipitate line was obtained in the reaction of the enzyme with rabbit antibody (Fig. 2). Despite nearly complete loss of activity the precipitate line could be detected using the corresponding substrates as specific stains for the enzyme protein.

Our previous studies have shown that these enzymes were from the soluble fraction of the cytoplasm (non-mitochondrial aspartate transaminase) (15).

Fig. 1

Sedimentation patterns of nonmitochondrial aspartate transaminase. The protein concentration was 15 mg/ml, 0.1M phosphate buffer, pH 7.5. The photographs were taken at 16 min. intervals after full speed was attained

Fig. 2

Precipitate lines of nonmitochondrial aspartate transaminase in immunoelectrophoresis

Nonmitochondrial aspartate transaminase (0.5 mg/ml) was filled into the wells; the trough contained specific anti-aspartate transaminase antibody. After the lines became visible the agar gel was washed in physiologic saline solution for several hours. The gel was separated at the trough; the lower part in the picture was stained for aspartate transaminase, and the upper part as a control was incubated with the same staining solution without L-aspartate. The lower line exhibited specific staining qualities of the enzyme protein

Tab. 1

The relation of iodination to enzyme activity

0.93 mg protein in 1 ml phosphate buffer 0.1M pH 7.5 was used in each experiment

The iodination solution consisted of a mixture of 1) 0.66 mg free iodine in 2.5 ml KI 0.002M, 2) 130 μC Na^{131}I in 0.74 ml 0.1N NaOH, and 3) 1.1 ml 0.1N HCl

The pH of the solution after iodination was between 7.2 and 7.8

Added iodination solution [ml]	Iodine bound after precipitation with trichloroacetic acid [%]	Iodine bound following dialysis [%]	Iodine bound per molecule [atoms]	Loss of enzyme activity [%]
0.06	0.5	0.7	0.04	0
0.10	0.67	0.8	0.1	0
0.15	0.47	0.52	0.1	0
0.2	1.3	1.47	0.35	6
0.25	2.1	2.14	0.65	12
0.35	3.0	3.0	1.2	28
0.4	3.2	3.6	1.7	44
0.5	3.7	3.75	2.4	60
0.75	4.4	—	3.9	77
1.0	4.9	—	5.9	85

Effect of iodination on enzyme activity

The results are given in Table 1. They indicate that the activity is lost in proportion to the number of iodine atoms incorporated per molecule. Whereas the activity is retained with 0.35 iodine atoms, a marked inhibition could be observed with 1—2 iodine atoms per molecule. In the range from 2 to 6 atoms of iodine bound per molecule of enzyme, approximately 60—85% of the original activity was lost.

Figure 3 shows the behavior of nonmitochondrial aspartate transaminase in chromatography on DEAE-Sephadex. Generally the peak of the eluted aspartate transaminase is not sharp, and the protein can be gradually separated from DEAE-Sephadex by increasing the molarity of the buffer. It was found in the various peaks that an increase in molarity causes a shift in the proportion of radioactivity to enzyme activity in favour of the former; this suggests either the presence of traces of free iodine, which would be eluted only by increasing the molarity from 0.008M, pH 7 to 0.05M, pH 6, or by some inhomogeneity of the variably iodinated molecules of the nonmitochondrial aspartate transaminase during the iodination procedure.

The effect of iodination on the affinity of the molecules for their substrates

The alteration of the MICHAELIS constant was estimated for two substrates by keeping the level of one constant, as described under methods. In Table 2 the results of

many experiments are summarized for the native and iodinated enzymes (for nonmitochondrial aspartate transaminase more than 1.2 iodine atoms per molecule). Statistical analysis of these results shows a significant

Fig. 3

Column chromatography of labelled aspartate transaminase on DEAE-Sephadex with discontinuous change of buffer. 5.15 mg aspartate transaminase with 0.7 iodine atom per molecule was separated on the column. The loss of activity due to labelling was 26%, and the recovery of the enzyme activity amounted to 66%. The volume of each fraction was 5 ml

Tab. 2

MICHAELIS constants of the native and labelled enzyme. Standard deviation (1 S.D.) in parentheses

For nonmitochondrial aspartate transaminase MORINO and coworker (45) found a MICHAELIS constant of 0.3 mM for 2-oxoglutarate and of 5 mM for L-aspartate

	2-oxoglutarate (mM)	L-aspartate (mM)
Km for aspartate transaminase	0.28 (± 0.07) n = 12	8.6 (± 1.1) n = 9
Km for labelled aspartate transaminase (= or > 1.2 iodine atoms per molecule)	0.14 (± 0.05) n = 7	18.2 (± 7.4) n = 9
	P < 0.02	P < 0.001

alteration of the MICHAELIS constant for two substrates of aspartate transaminase after iodination, i.e. an increasing affinity for 2-oxoglutarate and a reduced affinity for aspartate.

Effect of iodination on the immunological properties of the enzyme

The percentage of immune inhibition of nonmitochondrial aspartate transaminase by specific antibodies is given in Table 3. From this it can be seen that the decrease of inhibition of enzyme activity as iodination is increased, as compared with the control, is not of great significance and probably always appears in very high iodination. Quantitative measurement of variably iodinated aspartate transaminase using MANCINI's precipitation method, as shown in Table 4, did not reveal any alteration of the capacity of the enzyme to bind antibody with iodination up to 2.4 iodine atoms per molecule.

Tab. 3

Immune inhibition of native and variously labelled aspartate transaminase by incubation of the enzyme with a constant amount of antibody excess (0.015 ml)

Iodine [atoms per molecule]	Loss of enzyme activity by iodination [%]	Immune inhibition of 18 mU native enzyme by a constant amount of antigen [%]
0 (control)	—	79
0.04	0	72
0.1	0	73
0.35	6	66
0.65	12	68
1.2	28	76
1.7	44	70
2.4	60	70

Tab. 4

Influence of iodination on the antigen-antibody binding capacity in agar gel after MANCINI

Iodine [atoms]	Antigen amount [μg]	Surface of precipitate [mm^2]	Index = surface of precipitate to antigen amount
0.1	0.865	89	102
0.35	0.793	80.5	101
1.2	0.704	74.5	105
2.4	0.63	66	104

Fig. 4

Electrophoretic mobility of native (above) and labelled aspartate transaminase (below) in starch gel after SMITHIES. The amount of protein applied was 5.6 μg above and 14.7 μg below, with 7.5 iodine atoms per molecule, 0.3M borate buffer, 0.06M NaOH, pH 8.2. The gel length was 22 cm, the voltage 500 V, migration time 7.5 hours at 4°. The enzyme was detected by a aspartate transaminase specific staining method

Gel electrophoresis

As shown in Figure 4 for nonmitochondrial aspartate transaminase, the protein migrates in starch gel in several closely spaced bands which have many properties in common, according to the experiments of MARTINEZ-CARRION and coworkers (32). In the view of ARRIO-DUPONT and coworkers (33) these multiple forms of nonmitochondrial aspartate transaminase do not represent aggregation or dissociation of subunits, but are rather the result of conformation changes after oxidation of certain SH-residues. In polyacrylamide gel, however, the aspartate transaminase protein migrates as a sharp, homogenous band between the albumin and transferrin zones. The aspartate transaminase proteins labelled with up to 7.5 atoms of iodine per molecule show the same electrophoretic mobility as the native enzyme.

Fate of labelled enzyme in the animals

Figure 5 shows the course of the enzyme activity and protein-bound radioactivity in the sera after intravenous and subcutaneous injection. Discrepancies between these two parameters could not be verified.

a)

b)

Fig. 5

Behavior of enzyme activity (•-•-•) and protein-bound radioactivity (o-o-o) in the sera of two piglets after injection of 0.5 to 1 mg of labelled aspartate transaminase (5.7 iodine atoms per molecule) and with 5 mg of native enzyme. a) after subcutaneous injection; b) after intravenous injection. The enzyme activity in the sera of the piglets before injection (25-40 U/l) was deducted from the measured activity values

Discussion

In the course of labelling with ^{131}I two processes are involved: the changes caused by radiation damage and the chemical reactions brought about by the labelling procedure. According to the results of various research groups (34), the specific activity below which radiation damage occurs at an insignificant rate is widely accepted as being 5–10 $\mu\text{C}/\text{mg}$ of protein.

In the chemical reaction the hydrogen atom on the tyrosine ring and possibly on that of histidine was substituted by iodine (35, 36). Oxidation of cysteine, methionine, tryptophan, serine and threonine can occur (37).

Further possible alterations of the molecule due to iodination are not known in detail.

The loss of enzyme activity through the labelling process is closely associated with alteration of the active center itself, or of some other part of the enzyme which is important for the enzymatic function. The loss might be due to substitution of iodine on a tyrosyl residue intimately involved in the enzyme activity. Iodination of lactate dehydrogenase produces an inhibition of enzyme activity which is reversible by incubation with cysteine (11). This suggests that the SH-groups are oxidised during the labelling procedures. However, iodination of aldolase and loss of enzyme activity was not due to an oxidative reaction, but rather to the substitution of iodine on to a tyrosyl residue which is involved in the catalytic activity of aldolase (13). On the other hand, we were not able to observe any reactivation of 0.2 mg of nonmitochondrial aspartate transaminase, whose activity had been reduced to 38% by labelling with iodine, after incubation for 3 hrs with 1 mM reduced glutathione.

The structure of nonmitochondrial aspartate transaminase is not clear. POLYANOWSKI and coworker (38) found 6–7 SH-groups in this molecule, two of which were responsible for the enzyme activity. The active site of the enzyme may be a fragment consisting of Ser-(phosphopyridoxyl)Lys-Asp-Phe (39). Transamination between pyridoxal phosphate and amino acids takes place through multiple steps, for which a histidine residue, whose proximity to the active site, is not in

the primary sequence (40), and a tyrosine residue, which appears to be involved in coenzyme binding, are essential to catalysis (41). It is interesting that the photo-oxidation which inactivates aspartate transaminase through the destruction of histidine residue, does not cause any alteration of over-all protein tertiary structure, as judged by ultracentrifugation, optical rotatory dispersion and complement fixation techniques. The inactivated enzyme could bind the substrate, but could not convert these to products (42, 43). Our results indicate that oxidation of SH-groups is not essential for inhibition of enzyme activity. Substitution of a tyrosyl residue outside of the active site with iodine may be involved in this phenomenon.

According to alteration of the MICHAELIS constant during the iodination procedure, the affinity of the enzyme increases for 2-oxoglutarate and decreases for L-aspartate. It is not clear whether this alteration is caused by the presence of the iodine as an "allosteric effector" in the sense of the MONOD-WYMAN-CHANGEUX model (44), or by superficial structural changes. Since the immunological properties of the enzyme, i. e., its inhibition by antibodies and its capacity for binding antibodies, are hardly affected, it can be concluded, as inactivation of the enzyme by photooxidation showed, that the alterations do not involve any essential modification of the tertiary structure responsible for the antigen specificity. The similar electrophoretic mobility in polyacrylamide gel and in starch gel and their similar rates of disappearance from the vascular space, as compared to the nonlabelled enzyme, support these findings.

Our results concerning nonmitochondrial aspartate transaminase show that not only must a loss of biological activity be reckoned with in the process of iodination, but that some alteration of the affinity of the enzymatically active molecules to their substrates also occurs. These alterations of the functional integrity need not, however, be interpreted as an essential modification or deformity of the total structure.

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Prof. Dr. S. Massarrat
Medizinische Universitäts-Poliklinik
355 Marburg/Lahn
Germany