Role of Tyrosine Residues in Mitochondrial Aspartate Aminotransferase from Beef Kidney†

Roberto Scandurra,*,‡ Giovanni Polidoro, Domenico Di Cola, Laura Politi, and James F. Riordan

ABSTRACT: Mitochondrial aspartate aminotransferase from beef kidney is 50% inhibited after 2 hr treatment with 2.5 m*M* tetranitromethane at pH 8. Two tyrosine residues per enzyme protomer (46,000 daltons) are modified by the reagent either in the holoenzyme or in the apoenzyme. In both cases the five SH groups titratable with *p*-mercuribenzoate are not modified by the reagent. However, with a tetranitromethane concentration higher than 2.5 m*M* and 10 m*M* mercaptoethanol, an additional tyrosine residue is nitrated in both holo- and apoenzymes. These results are not affected by the presence in the incubation mixture of the substrates α-ketoglutarate and glutamate both at ten times their K_m values. Mercaptoethanol does not impair the recombination of native or nitrated apoenzyme with the coenzyme and does not reduce the coenzyme moiety of native or nitrated holoenzyme, but promotes a conformational change in the nitrated holoenzyme which causes inactivation. Hydrosulfite promotes the reduction of the coenzyme moiety of native and nitro holoenzyme resulting in their inactivation, largely in the nitrated form. The recombination of the coenzyme with native or nitrated apoenzyme is not influenced by hydrosulfite.

The role of tyrosine residues in cytoplasmic aspartate aminotransferase from pig heart has been pointed out by several investigators (Turano et al., 1968, 1971; Christen and Riordan, 1970; Shlyapnikov and Karpeisky, 1969). In particular Christen and Riordan (1970) and later Birchmeier et al. (1973b) demonstrated that nitration of one tyrosyl residue occurs rapidly only in the presence of both substrates, i.e. during catalysis. The term "syncatalytic" nitration was introduced by these authors to define this unusual situation. Turano et al. (1971) demonstrated that the apoenzyme is also highly reactive to nitration, with the same tyrosine residue likely being nitrated as in the holoenzyme in the presence of substrates.

We have been interested in the study of the isozyme of aspartate aminotransferase extracted from beef kidney mitochondria (Scandurra and Cannella, 1972). This isozyme, with a mol wt of 93,000, is composed of two 46,000 mol wt protomers, probably not identical. It contains 13 tyrosine and 7 cysteine residues per protomer, 5 of the latter being titratable with *p*-mercuribenzoate. This enzyme appears to be structurally different from the homologous isozyme isolated from pig heart, but nevertheless it exhibits very similar kinetic parameters, spectra, and coenzyme content (Scandurra and Cannella, 1972). A preliminary investigation (Polidoro et al., 1973a) has suggested that tyrosine residues might be involved in the catalytic activity of the enzyme.

The present paper is a more detailed study of the role played by the tyrosine residues in the activity of this isozyme, and an analysis of certain differences between it and the two isozymes from pig heart, in order to better understand the relationship between structure and function of enzymes catalyzing the same reaction.

Experimental Section

Materials. Mitochondrial aspartate aminotransferase, containing more than 90% of the α subform and having a specific activity of about 120 units, was prepared from beef kidney cortices as described previously (Scandurra and Cannella, 1972). The same work describes the preparation of apoenzyme. Malate dehydrogenase, α-ketoglutaric acid, and NADH were purchased from Boehringer (Mannheim); *p*-hydroxymercuribenzoate was from Sigma Chemical Co.; pyridoxal 5′-phosphate was from Calbiochem; and Bio-Gel P-6 was a Bio-Rad product. Tetranitromethane was from Fluka (Buchs); before use it was washed three times with distilled water and diluted with 96% ethanol. All other chemicals were reagent grade products from Merck (Darmstadt).

Methods. Nitration of 1×10^{-5} *M* enzyme was performed as described by Sokolovsky et al. (1966) either in 0.05 *M* Tris-Cl or in 0.05 *M* potassium phosphate buffer (pH 8) at room temperature for 2 hr. For nitration of the apoenzyme, phosphate buffer was preferred in order to avoid the partial precipitation of protein that occurs when Tris-Cl is used. Samples were diluted 200-fold with one of the buffers and 0.1 ml of this solution assayed for activity was determined essentially according to the method of Karmen (1955) with the slight modification of Scandurra and Cannella (1972). Excess reagent was removed by gel filtration through a column (25 × 1.5 cm) of Bio-Gel P-6 equilibrated with 0.05 *M* Tris-Cl buffer (pH 8).

Nitrotyrosine content was determined by amino acid analysis of the protein hydrolyzed in 6 *N* HCl at 110° for 24 hr, using an Optika (Milano) Aminolyzer, and by spectrophotometry following conversion of the enzyme to the pyridoxamine form and using E_{428} = 4100 (Sokolovsky et al., 1966). For the determination of tryptophan, hydrolysis was performed in *p*-toluenesulfonic acid (Liu and Chang, 1971).

† From the Institute of Biological Chemistry, University of Chieti, Chieti, Italy, and Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115. *Received January 22, 1975.* This work is part of a program supported by Italian Consiglio Nazionale delle Richerche.

‡ Present address: Istituto Chimica Biologica, Città Universitaria, 00185-Rome, Italy.

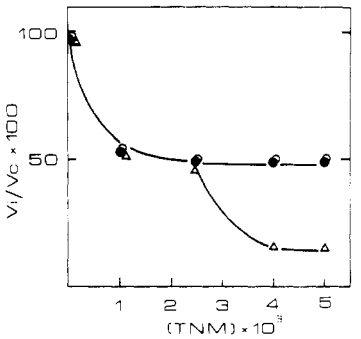

FIGURE 1: Inhibition of holoenzyme (○) and apoenzyme (●) of mitochondrial aspartate aminotransferase from beef kidney by tetranitromethane. Holoenzyme (1×10^{-5} *M*) either in 0.05 *M* Tris-Cl (pH 8) or in 0.05 *M* phosphate buffer (pH 8) and apoenzyme (1×10^{-5} *M*) in 0.05 *M* phosphate buffer (pH 8) were incubated at 20° with tetranitromethane at the concentrations reported on the abscissa, for 2 hr; the incubation mixtures were 200-fold diluted in the same buffers and 0.1 ml was assayed for residual activity (reported as the ratio of that of the modified enzyme, V_i, and the unmodified control, V_c, times 100). Recombination of apoenzyme was performed at 38° for 1 min with a 100-fold molar excess of pyridoxal 5′-phosphate. The activity of samples containing 1×10^{-5} *M* holoenzyme incubated for 2 hr with different concentrations of tetranitromethane, at which time 2-mercaptoethanol was added to give a concentration of 10 m*M*, is reported (△). Activity was measured 30 min after the mercaptoethanol addition.

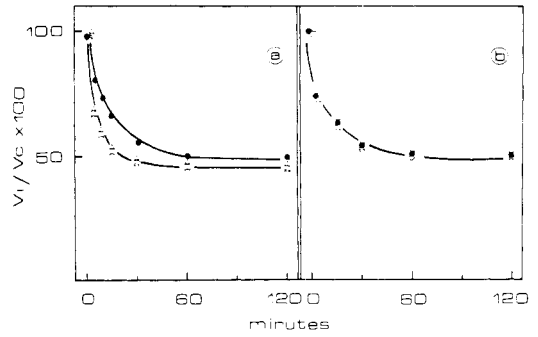

FIGURE 2: Effect of substrates on the inhibition exerted by tetranitromethane on holoenzyme (a) and apoenzyme (b). Enzyme (1×10^{-5} *M*) either in Tris-Cl or in phosphate buffer (0.05 *M*, pH 8) was incubated at 20° with 2.5 m*M* tetranitromethane in the absence (●) and in the presence of either 2.5×10^{-2} *M* α-ketoglutarate (△), 5×10^{-2} *M* glutamate (□) or both (○). Symbols and concentrations for the apoenzyme (●) (part b) in 0.05 *M* phosphate buffer (pH 8) are the same as in part a. The activity is expressed as in Figure 1.

Protein concentration was estimated either spectrophotometrically using a molar absorptivity of 1.25×10^5 M^{-1} cm.$^{-1}$or by amino acid analysis adding a known amount of norleucine as internal standard prior to hydrolysis. Optical rotatory dispersion spectra were obtained with a Cary 60 recording spectropolarimeter: the data are reported as mean residue rotation, *m′*, as suggested by Fasman (1963).

Sulfhydryl groups were determined with *p*-mercuribenzoate as described by Boyer (1954) in 0.2 *M* potassium phosphate buffer (pH 7); the protein was added to a 15-fold molar excess of the reagent and after standing overnight the free reagent was back-titrated with cysteine. Reconstitution of the apoenzyme was obtained with a 1-min incubation at 38° using a 100-fold molar excess of pyridoxal 5′-phosphate. The binding capacity of the apoenzyme was estimated by fluorescence quenching (Scandurra and Cannella, 1972) using an Aminco Bowman spectrofluorometer. Absorption spectra were recorded with a Cary 14 spectrophotometer.

Table I: Reaction of Mitochondrial Aspartate Aminotransferase from Beef Kidney with Tetranitromethane in the Presence and Absence of Substrates; Amino Acid Modifications.

Enzymes	Substrate Added[a]	Inhibition (%)	Cys Residues Titrated per Protomer[b]	Tyr Residues Modified per Protomer[c]	
				+SH[d]	−SH
Holo-	None	50	5	2.9	2.0
Holo-	Glutamate + α-ketoglutarate	50	5	2.9	
Apo-	None	50	5	3.1	2.0
Apo-	Glutamate + α-ketoglutarate	50	5	3.1	

[a]Both at ten times their K_m values. [b]Back-titration with *p*-mercuribenzoate as reported in the Experimental Section. [c]Determined as nitrotyrosines by amino acid analysis, following removal of reagents by gel filtration. [d]2-Mercaptoethanol (10 m*M*) was added at the end of nitration; the aliquots were taken for hydrolysis 30 min after this addition to ensure complete reduction of any cystine residues.

Results

Reaction of Holo- and Apoenzyme with Tetranitromethane, in the Presence and Absence of Substrates. Incubation of the holoenzyme (either in 0.05 *M* Tris-Cl buffer (pH 8) or 0.05 *M* phosphate buffer (pH 8)) and the apoenzyme (in 0.05 *M* phosphate buffer (pH 8)) of mitochondrial aspartate aminotransferase from beef kidney with tetranitromethane for 2 hr brings about a 50% reduction in activity as shown in Figure 1. Increasing the concentration of tetranitromethane above 2.5 m*M* does not bring about either an appreciable additional inactivity or a significant increase in the extent of tyrosine modification. Therefore, 2.5 m*M* reagent has been chosen for the experiments in the present paper. The effect of 2-mercaptoethanol, shown in this figure, is discussed below.

The effects of substrates, i.e. α-ketoglutarate and glutamate, separately or together, on holo- and apotransaminase, are presented in Figure 2. The substrates, at concentrations 10 times their K_m values (Scandurra and Cannella, 1972), are without effect on the initial rate of inactivation of the apoenzyme, while they increase the rate of the holoenzyme from a factor of 2.2 to 3.4×10^{-2} min^{-1}. This effect is more readily apparent when the molar excess of tetranitromethane with respect to the enzyme is very high (10^4-fold). Nevertheless, the extent of inactivation, in both cases, is not more than that obtained in the absence of substrates, i.e. 50%. As reported in Table I, amino acid analysis reveals that 2.9 tyrosine residues are modified per protomer in the holoenzyme and 3.1 in the apoenzyme. The same results are found when nitration is performed in the presence of substrates. These amino acid analyses were performed on nitrated samples to which 10 m*M* 2-mercaptoethanol had been added in order to reduce any cysteine residues which might have been oxidized to cystine by tetranitromethane. If the 2-mercaptoethanol is omitted, the number of nitrotyrosines detected is somewhat less, i.e. about two per protomer in each case. The back-titration of SH groups with *p*-mercuribenzoate shows (Table I) that all of the titratable SH groups found in the native enzyme are found in the nitrated enzyme as well. Nevertheless, some other experi-

Table II: Inactivation of Mitochondrial Aspartate Aminotransferase from Beef Kidney Treated with Tetranitromethane as a Function of pH (Incubation Conditions as Described in the Text).

pH[a]	$V_i/V_c \times 100$[b]	pH[a]	$V_i/V_c \times 100$[b]
6	82	8	73
7	77	9	67

[a] pH 6, 7, and 8 were obtained with phosphate buffers, and pH 9 with glycine–NaOH buffer; each buffer was 0.05 *M*. [b] Residual activity, measured after 30 min, is reported as the ratio of that of the modified enzyme, V_i, and the unmodified control, V_c, times 100.

ments have been performed to confirm that cysteine residues, in the present case, are not oxidized by tetranitromethane.

The oxidation of cysteine residues of proteins by tetranitromethane can occur both at pH 6 and at pH 8, whereas the nitration of tyrosine residues occurs only at pH 8 or above (Sokolovsky et al., 1966). Since the activity of the beef kidney enzyme is strictly dependent on the integrity of SH groups, as shown by the experiments in Figure 3 with *p*-mercuribenzoate, lowering the pH should favor cysteine oxidation by tetranitromethane with a consequent decrease of the activity. As reported in Table II, just the opposite results are obtained. As the pH is lowered, there is less inactivation.

Furthermore, when the enzyme is treated at pH 7 with a 100-fold molar excess of *p*-mercuribenzoate, all the activity, as shown in Figure 3, is lost in 2 hr. The rate of inactivation is not increased by the simultaneous addition of tetranitromethane. Moreover, the activity of the sample treated with *p*-mercuribenzoate alone is restored completely, at any time, by addition of 10 m*M* 2-mercaptoethanol. However, those of the samples treated with both *p*-mercuribenzoate and tetranitromethane, when measured immediately after addition of mercaptoethanol, return only to the values found for the control sample, i.e. the enzyme treated with tetranitromethane alone. Finally, there is no modification of methionine or tryptophan residues during the nitration process.

Properties of Nitrated Holo- and Apoenzymes. Neither the holo- nor the apoenzyme undergoes any gross conformational change as a consequence of nitration. The optical rotatory dispersion (ORD) curves of the native and nitrated holo- and apoenzymes in the 200–300-nm region are identical as shown in Figure 4.

The absorption spectra of the native and nitrated holoenzymes and those of the holoenzymes reconstituted from either the apo native or the apo nitro enzyme, in 0.05 *M* Tris-Cl (pH 8), show a peak centered at 360 nm characteristic of the pyridoxal form of the transaminase. In order to assay the quantitative reconstitution of the nitrated apoenzyme with coenzyme, the fluorometric method used by Scandurra and Cannella (1972) has been used. Based on the quenching of apoenzyme fluorescence emission at 335 nm (excitation at 280 nm) on titration with the coenzyme, a quantitative evaluation of the amount of coenzyme bound by the apoenzyme can be determined. Titration of native and nitrated apoenzymes with pyridoxal 5′-phosphate shows similar results, demonstrating that the amount of coenzyme bound by the nitrated apoenzyme and by the native apoenzyme is the same, i.e. 1 mol per enzyme protomer. Kinetic measurements have shown that the K_m values of the native and reconstituted holoenzymes for aspartate and α-ketoglutarate

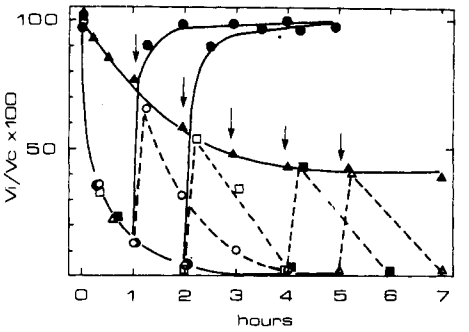

FIGURE 3: Reactivation of the holoenzyme treated in phosphate buffer (0.05 *M*, pH 7) with 2.5 m*M* tetranitromethane (▲), 1 m*M* *p*-mercuribenzoate (●), and by both (○, □, ■, △). Samples containing 1×10^{-5} *M* enzyme were incubated with the reagents and, at the times indicated by arrows, 10 m*M* 2-mercaptoethanol was added. Residual activity is measured and expressed as in Figure 1.

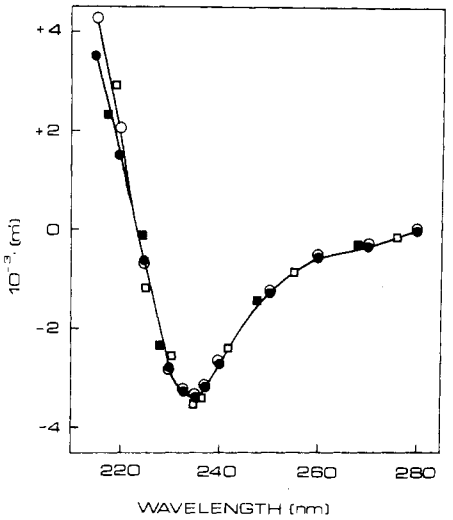

FIGURE 4: Optical rotatory dispersion curves of native (●) and nitrated (○) holoenzymes and native (■) and nitrated apoenzymes (□). Protein concentration was in each case 5.4×10^{-6} *M*. Spectra of holoenzymes were measured in 0.05 *M* Tris-Cl; spectra of apoenzymes were measured in 0.05 *M* phosphate buffer. Both were measured at pH 8 with 1-mm path length cuvets at 20°; *m′* is mean residue rotation.

are not altered in the nitrated and reconstituted nitrated apoenzymes. The V_{max} values in these two latter cases are decreased by about 50%.

Effect of Reducing Agents. The effects of two reducing agents, 2-mercaptoethanol and hydrosulfite, on the nitrated enzyme have been studied; the first has been used in order to reduce any cystine residues formed as a side reaction during the nitration process and the second to reduce nitrotyrosines to aminotyrosines (Sokolovsky et al., 1967). The addition of 10 m*M* 2-mercaptoethanol to samples containing holoenzyme and tetranitromethane, the latter in concentrations ranging from 0 to 2.5 m*M*, followed by removal of reagents through gel filtration, is without effect either on the activity, as shown in Figure 1, or on the spectrum in the region of coenzyme absorption. When the tetranitromethane concentration is higher than 2.5 m*M*, the addition of 2-mercaptoethanol at the end of nitration promotes a large inactivation of the enzyme, nevertheless without affecting its absorption at 360 nm.

The addition of hydrosulfite, which was found devoid of sulfite, to the nitrated holoenzyme abolishes the absorbance at 428, markedly decreases that at 360 nm, and, as shown in

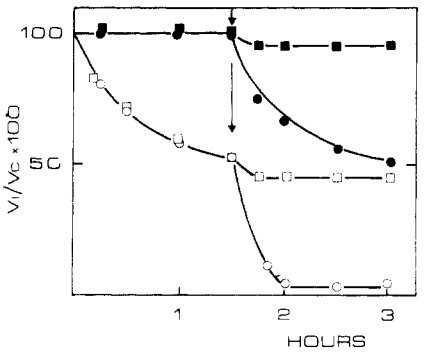

FIGURE 5: Effect of hydrosulfite on native (●) and nitrated (○) holoenzymes (in 0.05 *M* Tris-Cl buffer (pH 8)) and on native (■) and nitrated (□) apoenzymes (in 0.05 *M* phosphate buffer (pH 8)). Enzyme, in each case 1×10^{-5} *M*, was incubated with 2.5 m*M* tetranitromethane; at arrows 5 m*M* hydrosulfite was added. Activity is expressed as in Figure 1.

Figure 5, totally inactivates the enzyme. Inactivation also occurs on treating the native holoenzyme with hydrosulfite, though it is slower and less extensive than for the nitrated holoenzyme. In contrast, the reconstitution of native and nitrated apoenzymes, which in both cases occurs to about 50%, is virtually unimpaired by their prior treatment with hydrosulfite.

Discussion

Tetranitromethane is considered to be a useful reagent for the chemical modifications of tyrosine residues in proteins. Using this reagent on the mitochondrial holo and apo forms of aspartate aminotransferase from beef kidney, we have found that 2 to 3 tyrosine residues out of a total of 13 can be modified per protomer.

The presence of substrates, i.e., α-ketoglutarate and glutamate, at concentrations 10 times their K_m values, does not change these results. Either in the holoenzyme or in the apoenzyme the maximal inactivation obtained with 2.5 m*M* tetranitromethane is about 50%. Neither the presence of substrates, nor increasing the reagent concentration to 5 m*M*, nor increasing the incubation time beyond 2 hr brings about a greater extent of inactivation. From these results it is possible to conclude that in this aminotransferase the "syncatalytic inactivation" found by Christen and Riordan (1970) on the cytoplasmic isozyme from pig heart, i.e. the nitration of tyrosine residues only during catalysis, is absent. It should be noted, however, that the rate of reaction with tetranitromethane is indeed enhanced by the presence of substrates (Figure 2).

The spectral properties of the nitrated holoenzyme rule out any effect on the coenzyme moiety; it remains in the PLP form. Furthermore, binding of the coenzyme to the nitrated apoenzyme is unimpaired.

The nitration of two tyrosine residues per holoenzyme or apoenzyme protomer, or of three tyrosine residues when 2-mercaptoethanol is added, does not induce gross conformational changes either in the holo- or in the apoenzyme as evidenced by the close similarity of the ORD curves for the native and modified enzymes (Figure 4).

The side effects of tetranitromethane on cysteine, tryptophan, and methionine residues described by others (Cuatrecasas et al., 1968; Sokolovsky et al., 1970) have not been found in the present enzyme. The five titratable SH groups per protomer present in the native enzyme are not modified by the reagent and no significant modification of tryptophan and methionine residues has been detected.

From the results presented it can be concluded that this aminotransferase shows a behavior toward tetranitromethane different from that presented by the soluble aspartate aminotransferase from pig heart. In the latter the nitration of one tyrosine per holoenzyme protomer is strictly dependent on the presence of substrates, i.e. is "syncatalytic", while in the apoenzyme tyrosyl nitration is quite facile (Turano et al., 1971). Originally, and using the commercially available enzyme, the presence of substrates induced nitration of only 0.5 tyrosyl residue per protomer (Christen and Riordan, 1970). Later, using the purified α subform of the pig heart enzyme, it was shown that 1.0 tyrosyl residue per protomer is nitrated under these conditions (Birchmeier et al., 1973b). Moreover, nitration of this tyrosine is not syncatalytic per se but requires the prior oxidation of one specific cysteinyl sulfhydryl group, that of Cys-390 (Birchmeier et al., 1972; Ovchinnikov et al., 1973) which could not be detected in the commercial enzyme preparation. It is now believed that syncatalytic inactivation of the pig heart cytosol enzyme is due to sulfhydryl oxidation while the subsequent tyrosyl nitration fixes the coenzyme in the pyridoxamine form. These results are consistent with the conclusion that in the pig heart cytoplasmic enzyme the coenzyme masks a tyrosyl phenolic group which is nitrated easily in the apoprotein or in the holoenzyme as the substrate–enzyme complex is formed. In the present case, since there is no difference in nitrotyrosine content between the holo- and apoenzymes, it would seem that in the holoenzyme the coenzyme does not impair nitration of the two tyrosine residues, which are probably the same as those nitrated in the apoenzyme. Furthermore, no tyrosine residues appear to be involved in binding coenzyme to the apoenzyme, whereas in the cytoplasmic enzyme from pig heart, nitration of the particularly reactive tyrosine residue totally prevents the recombination of the coenzyme and apoenzyme (Turano et al., 1971).

The effect exerted by reducing agents can provide additional information about the topography of the active site of the mitochondrial aminotransferase which appears to be different from that of the cytoplasmic isozyme from pig heart. In the mitochondrial isozyme 2-mercaptoethanol is unable to perturb the absorption spectrum in the region of coenzyme absorbance. Nevertheless, the extent of nitration is dependent on the presence of the reducing agent, and the degree of inactivation, which occurs in the presence of 2-mercaptoethanol, increases with increasing tetranitromethane concentration.

Since the coenzyme itself is not affected by 2-mercaptoethanol, the effect of the reducing agent should be chemical or conformational. In fact, addition of excess 2-mercaptoethanol to the enzyme while tetranitromethane is still present could generate the sulfenyl nitrate derivative, $HOCH_2CH_2SNO_2$. This can react with a variety of amino acid side chains including cysteine but it can also react with a second molecule of 2-mercaptoethanol to give the oxidized dimeric form and liberate nitrate. Some nitrous acid may be generated in this way leading to the observed inactivation.

This explanation has been ruled out since addition to the incubation mixture of 5 m*M* nitrate, before or after gel filtration, is unable to further inactivate the nitrated enzyme. The effect of the reducing agent could then be ascribed to a local change in protein conformation, not detectable by ORD, which promotes nitration of a third tyrosine residue. When nitration is performed with a tetranitromethane con-

centration higher than 2.5 m*M* in the presence of 2-mercaptoethanol, the resulting conformational change promotes a large inactivation of the enzyme which is not detected when 2-mercaptoethanol is added to a nitrated enzyme devoid of excess reagent by gel filtration. A similar explanation can be used for the experimental results reported in Figure 3; the rapid decrease in enzyme activity following the initial reactivation exerted by 2-mercaptoethanol upon the enzyme treated with both *p*-mercuribenzoate and tetranitromethane can be attributed to a denaturing effect of *p*-mercuribenzoate. These effects of *p*-mercuribenzoate have already been reported (Stankewitz et al., 1971). In the present case the reagent would appear to promote a conformational change of the protein with a resulting loss of enzymatic activity, this inactivation rate being independent of the time at which 2-mercaptoethanol is added, as shown in Figure 3.

The effect of hydrosulfite, on the other hand, is most probably exerted on the coenzyme moiety of the enzyme; its action appears to be different from that observed with the cytoplasmic isozyme from pig heart. With the latter, hydrosulfite reduces the nitrotyrosine residues to aminotyrosine but is without effect on the coenzyme moiety and, accordingly, on the enzyme activity (Christen and Riordan, 1970). In the present case, hydrosulfite exerts a double action: it reduces the nitrated tyrosines to aminotyrosines as demonstrated by the loss of absorption at 428 nm in both the holoenzyme and the apoenzyme and it reduces the coenzyme moiety. The complete inactivation of the nitrated holoenzyme by hydrosulfite is accompanied by a decrease in absorption at 360 nm demonstrating an effect of hydrosulfite on the coenzyme moiety of the enzyme. In support of this view, treatment of the native and nitrated apoenzymes with the reagent does not affect the subsequent binding of coenzyme as reported in Figure 5. Hydrosulfite inactivates native holoenzyme by about 50% just as it does the nitrated holoenzyme, but in this latter case the inactivation rate is higher; since hydrosulfite was found devoid of sulfite contamination, the inactivating effect is due to hydrosulfite itself. Hence, chemical modification of two tyrosines to either the nitro or amino derivatives favors the reduction of the coenzyme moiety of the protein by hydrosulfite.

From all the experimental data reported above, it is possible to conclude that the mitochondrial aspartate aminotransferase from beef kidney has two tyrosine residues per protomer, located in the proximity of the active site, that are selectively nitrated by tetranitromethane. No steric hindrance to the nitration is offered by the coenzyme. A third tyrosine residue, not important for the activity, is also nitrated if 2-mercaptoethanol is present. Nitration of these tyrosine residues does not impair coenzyme binding to the apoenzyme.

The behavior of the beef kidney mitochondrial enzyme toward tetranitromethane is quite dissimilar from that reported for the cytoplasmic isozyme from pig heart. The "syncatalytic" inactivation found for the cytoplasmic isozyme is not detectable with the kidney enzyme. Nevertheless, its rate of inactivation is doubled in the presence of substrates, though the actual extent of nitration and inactivation is not affected. This is in contrast to the 20-fold effect of substrates on the rate of inactivation of the pig heart cytoplasmic enzyme (Christen and Riordan, 1970). Nitration of the beef enzyme in the presence or absence of substrates reduces activity only by about 50%. Complete inactivation of the holoenzyme can occur but only under well-defined conditions through the combined action of tetranitromethane and the reducing agents; with 2-mercaptoethanol the coenzyme moiety of the enzyme is not affected, whereas with hydrosulfite the coenzyme is reduced. In the pig heart enzyme, tetranitromethane oxidizes thiols of Cys-45 and -82 of each protomer in the absence of substrate, reducing activity to about 60%, while the thiol of Cys-390 is oxidized syncatalytically concomitant with the nitration of one tyrosine residue and the reduction of activity to 3% (Birchmeier et al., 1973b). Cys-390 may not be absolutely essential to catalysis since it can be modified with cyanide with 60% retention of enzymatic activity (Birchmeier et al., 1973a). In the mitochondrial isozyme from beef kidney, the cysteine residues are not oxidized by tetranitromethane but instead the reagent nitrates two or three tyrosine residues not involved in the coenzyme binding. Importantly, for the cytoplasmic isozyme from pig heart, there is some evidence that a hydrogen bond between a tyrosine hydroxyl group and the pyridine nitrogen of pyridoxal 5′-phosphate figures prominently in the proposed mechanism of action (Braunstein, 1970; Ivanov and Karpeisky, 1969).

This sharp difference in behavior supports the idea that the active site of mitochondrial aspartate aminotransferase from beef kidney must be rather different from the active site of the cytoplasmic isozyme from pig heart, even though the kinetic parameters of the two enzymes are very similar.

It remains to be established whether or not the differences found are general differences between all cytoplasmic and mitochondrial isozymes of aspartate aminotransferase or whether they are due to the fact that these two isozymes in particular are extracted from different organs of different species. Some structural differences have, in fact, been found between the mitochondrial isozymes from beef kidney and pig heart (Scandurra and Cannella, 1972). For this reason, the cytoplasmic isozyme from beef kidney has been recently purified (Polidoro et al., 1973b) in order to extend the present study to isozymes from the same organ of the same species. Recently the reactivity of the cysteine and tyrosine residues of the aspartate aminotransferase from chicken heart cytosol has been examined (Kochkina and Torchinskii, 1975). In the absence of substrates a maximum of one tyrosyl residue per subunit was nitrated and one sulfhydryl group per subunit was oxidized with but a 10% loss of activity. When substrates were present one additional tyrosine was nitrated and one sulfhydryl oxidized while activity decreased to 65% of the control. Only a partial loss of activity occurred on blocking the sulfhydryl groups with *p*-mercuribenzoate in contrast to the 95% inactivation that occurs on similar treatment of the pig heart cytosol enzyme (Torchinskii and Sinitsina, 1970).

The corresponding mitochondrial enzymes from chicken and pig heart have also come under investigation (Gehring and Christen, 1975). One sulfhydryl group per subunit can be modified in each case with 5,5′-dithiobis(2-nitrobenzoate) without loss of activity. The presence of substrates increases the rate of reaction by one order of magnitude, but still enzymic activity is unchanged. Nitration of tyrosyl residues has not been examined with these enzymes; hence, a detailed comparison cannot yet be made. Still it would appear that some differences are apparent between the cytosol and mitochondrial enzymes from the same tissue even from the same species. One thing that all transaminases do seem to have in common is a change in reactivity of either tyrosyl or cysteinyl residues, or both, in response to the conformational adaptations that occur on formation of the enzyme–coenzyme–substrate compound. An increase rather than a

decrease in reactivity is the rule in these instances studied thus far. The mechanistic implications of this hyperreactivity are currently under investigation.

Acknowledgment

The authors are indebted to Dr. R. Spagnoli for carrying out the ORD spectra.

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