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Interaction of Aspartate Aminotransferase with Mercurochrome

RELATIONSHIP OF AN EXPOSED THIOL GROUP OF THE ENZYME TO THE ACTIVE CENTRE

By THEODORE G. KALOGERAKOS, NIKOLAOS G. OIKONOMAKOS, CONSTANTIN G. DIMITROPOULOS, IRENE A. KARNI-KATSADIMA* and ATHANASIOS E. EVANGELOPOULOS
The National Hellenic Research Foundation, 48, Vassileos Constantinou Avenue, Athens 501/1, Greece

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Mercurochrome strongly inhibits aspartate transaminase and 2,3-dicarboxyethylated aspartate transaminase. The native enzyme exhibits a biphasic time-course of inactivation by mercurochrome with second-order rate constants 1.62×10^{4} M$^{-1}\cdot$min^{-1} and 2.15×10^{3} M$^{-1}\cdot$min^{-1}, whereas the modified enzyme is inactivated more slowly (second-order rate constant 6.1×10^{2} M$^{-1}\cdot$min^{-1}) under the same conditions. The inhibitor inactivates native and modified enzyme in the absence as well as in the presence of substrates. Mercurochrome–transaminase interaction is accompanied by a red shift in the absorption maximum of the fluorochrome of about 10nm. Difference spectra of the mercurochrome–enzyme system versus mercurochrome, compared with analogous spectra of mercurochrome–ethanol, revealed that the spectral shifts recorded during mercurochrome–transaminase interaction are similar to those that occur when mercurochrome is dissolved in non-polar solvents. Studies of mercurochrome complexes with native or modified transaminase, isolated by chromatography on Sephadex G-25, revealed that native transaminase is able to conjugate with four mercurochrome molecules per molecule, but the modified enzyme is able to conjugate with only two mercurochrome molecules per molecule.

Although the primary structure of aspartate aminotransferase (L-aspartate–2-oxoglutarate aminotransferase, EC 2.6.1.1) has been determined (Ovchinnikov *et al.*, 1973; Doonan *et al.*, 1974), substantial work is still needed for the complete understanding of the structure–function relationship of its active centre. A number of amino acid residues have been implicated in the mechanism of action of transaminase. One lysine residue per subunit forms a Schiff base with pyridoxal phosphate and may function as a proton donor–acceptor during transamination (Hughes *et al.*, 1962; Braunstein, 1973). One histidine residue per subunit may also be involved in proton transfer (Martinez-Carrion *et al.*, 1967). Modification of one cysteine residue leads to 95% inactivation of the enzyme, its rate of modification being increased markedly by the presence of substrates (Birchmeier *et al.*, 1973). One tyrosine residue undergoes syn-catalytic modification (modification in the presence of substrates) (Christen & Riordan, 1970), and other data indicate that arginine residues are critical to the mechanism of action of transaminase (Riordan & Scandurra, 1975).

Cytoplasmic aspartate transaminase contains five thiol groups per monomer. Modification of the exposed residues cysteine-45 and cysteine-82 does not affect catalytic activity (Birchmeier *et al.*, 1973; Zufarova *et al.*, 1973). Cysteine-45 is the only thiol group of the enzyme that conjugates selectively with one maleate molecule (Polyanovsky *et al.*, 1973*a*). An increase in the titratable thiol groups of the enzyme after its interaction with aspartate has been observed spectroscopically by using substrate amounts of enzyme (Evangelopoulos & Sizer, 1968). The catalytic activity of transaminase is protected against *p*-chloromercuribenzoate by 2-oxoglutarate (Karni-Katsadima *et al.*, 1969). The enzyme very rapidly forms complexes with some derivatives of fluorescein, affecting both absorption- and emission-spectral characteristics of the fluorochromes and the catalytic activity of the enzyme (Dimitropoulos *et al.*, 1973).

In the present report the functional relationship between the active centre of the enzyme and the state of cysteine-45 is examined by studying the reactivity of dibromohydroxymercurifluorescein (mercuro-

* Present address: Hellenic Institute Pasteur, 127 Vassilissis Sofias Avenue, Athens 602, Greece.

chrome) with aspartate transaminase, in which cysteine-45 is free, and with aspartate transaminase partially *S*-2,3-dicarboxyethylated, in which cysteine-45 is conjugated with maleate. The ability of mercurochrome to form complexes with native or modified transaminases has been used to locate possible mercurochrome-binding sites and their effect on enzyme inactivation.

Experimental

Aspartate transaminase from pig hearts was prepared by the method of Jenkins *et al.* (1959) in succinate buffer (Turano *et al.*, 1964). The method was modified in the last step as described previously (Katsiris *et al.*, 1972). Partially 2,3-dicarboxyethylated enzyme was prepared by the original method of Jenkins *et al.* (1959) by using maleate buffer, and also by the later method of Polyanovsky *et al.* (1973*b*). Aspartate transaminase partially carboxymethylated and aspartate transaminase partially 2,3-dicarboxyethylated and carboxymethylated were obtained by the method of Zufarova *et al.* (1973). The enzyme preparations used in our studies had an A_{280}/A_{360} ratio around 10 and an A_{360}/A_{310} ratio of 2.5–3.0 at pH9.0. Calculations of transaminase molarity were based on a mol.wt. of 93400 (46700 per subunit) (Ovchinnikov *et al.*, 1973).

Mercurochrome, maleic anhydride, 2-oxoglutarate, L-aspartate, pyridoxal phosphate, pyridoxamine phosphate and Tris were obtained from BDH Chemicals, Poole, Dorset, U.K. Iodoacetate was from E. Merck, Darmstadt, Germany, *N*-ethylmaleimide was from Aldrich–Europe, Beerse, Belgium, 5,5′-dithiobis-2-nitrobenzoate) from Sigma Chemical Co., St. Louis, MO, U.S.A., and Sephadex various types was from Pharmacia, Uppsala, Sweden.

Transaminase activity was determined by measuring the A_{280} of the oxaloacetate formed by using an automatic Unicam SP.500 spectrophotometer. Kinetic measurements were obtained from a 3ml reaction mixture containing 100μmol of Tris/HCl buffer, pH8.0, and 20μmol each of L-aspartate and 2-oxoglutarate at 20°C, by the method of Cammarata & Cohen (1951). Absorption spectra were obtained with a Unicam SP.8000 recording u.v.–visible spectrophotometer. Fluorescence spectra of mercurochrome or of its reaction products with the enzyme were obtained with an Aminco–Bowman recording spectrofluorimeter.

Enzyme concentration was determined spectrophotometrically by using a molar absorption coefficient ε_{280} 130000 litre·mol^{-1}·cm^{-1} (Martinez-Carrion *et al.*, 1976). Native, 2,3-dicarboxyethylated and carboxymethylated transaminases were characterized by measurements of their activity and thiol-group content. Thiol groups were determined by the procedure of Ellman (1959) by using the molar absorption coefficient for 5-thio-(2-nitrobenzoate) ε_{412} 13600 litre·mol^{-1}·cm^{-1}.

Results

Physical and chemical characteristics of mercurochrome

It is known that the dissociated form of a thiol group, the mercaptide ion, is a far better nucleophile than the non-dissociated species (Lindley, 1960). Thus the thiol group of an organic molecule becomes less reactive as its dissociation is reversed with decreasing pH (Lindley, 1960; Polgar, 1974). It was therefore decided to examine spectroscopic and other properties of mercurochrome in relation to the optimal conditions of thiol reactivity.

Mercurochrome (also known as merbromin, mercurophage, chromargyre) has been used extensively for many years as a local antiseptic. It is freely soluble in water and water/ethanol mixtures and is practically insoluble in ethanol or acetone (*Merck Index*, 1968; White, 1920). Mercurochrome was selected for our studies because of its solubility and spectroscopic properties. The compound exhibits characteristic spectra with a sharp absorption maximum in the visible region (510nm), an area of the spectrum where neither transaminase nor its substrates absorb. Detailed spectroscopical studies performed as a function of pH demonstrated that the mercurochrome molecule in solution exhibits different equilibrium states between protonated and non-protonated forms. In Fig. 1(*a*) changes in the A_{510} ($\lambda_{max.}$) as a function of pH are presented. The pK values of the main equilibrium states calculated as described by Mercola *et al.* (1972) were pK_1 = 2.96, pK_2 = 4.45 and pK_3 = 6.6 (Fig. 1*b*). In the pH region 8–10 (near the pK value of many thiol groups), the A_{510} of mercurochrome is practically constant (Fig. 1).

Stability in the same pH region was also demonstrated for the emission spectra of mercurochrome, obtained by excitation at 508nm by using an Aminco–Bowman recording spectrofluorimeter. The optimal mercurochrome concentrations for the emission spectra were determined by studying the emission intensity of the solutions, at the emission maximum (528nm), as a function of the concentration of the fluorescein derivative. It was demonstrated that a linear relationship exists between emission at the maximum of the spectra and mercurochrome concentration for the region 0.1–1.1μM.

In another series of experiments the spectroscopic behaviour of mercurochrome during its interaction with various inorganic and organic sulphur compounds was examined. The difference spectra in Figs. 2(*a*) and 2(*b*) represent an example of the nature of the spectroscopic alterations that take place during the interaction of mercurochrome with sulphur

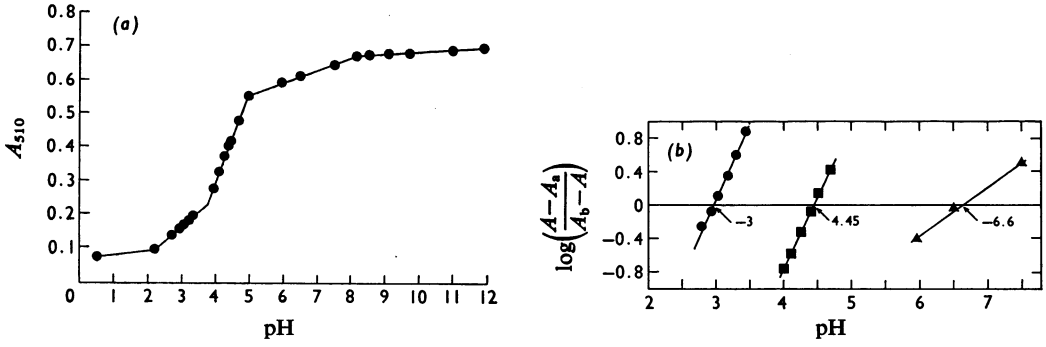

Fig. 1. *Changes in the absorbance of mercurochrome at 510nm with pH*

(*a*) Effect of pH on the maximum absorption of mercurochrome solutions (10μM) in 0.05M-sodium carbonate/bicarbonate, -Tris/HCl, -potassium phosphate, -sodium acetate, -HCl/phthalate and -HCl/KCl buffers. (*b*) Estimation of the apparent pK_1, pK_2 and pK_3 for the main equilibrium states of mercurochrome from A_{510} values. A is the absorbance at 510nm, A_a is the absorbance of the acid and A_b is the absorbance of the base form of mercurochrome for the respective equilibrium states.

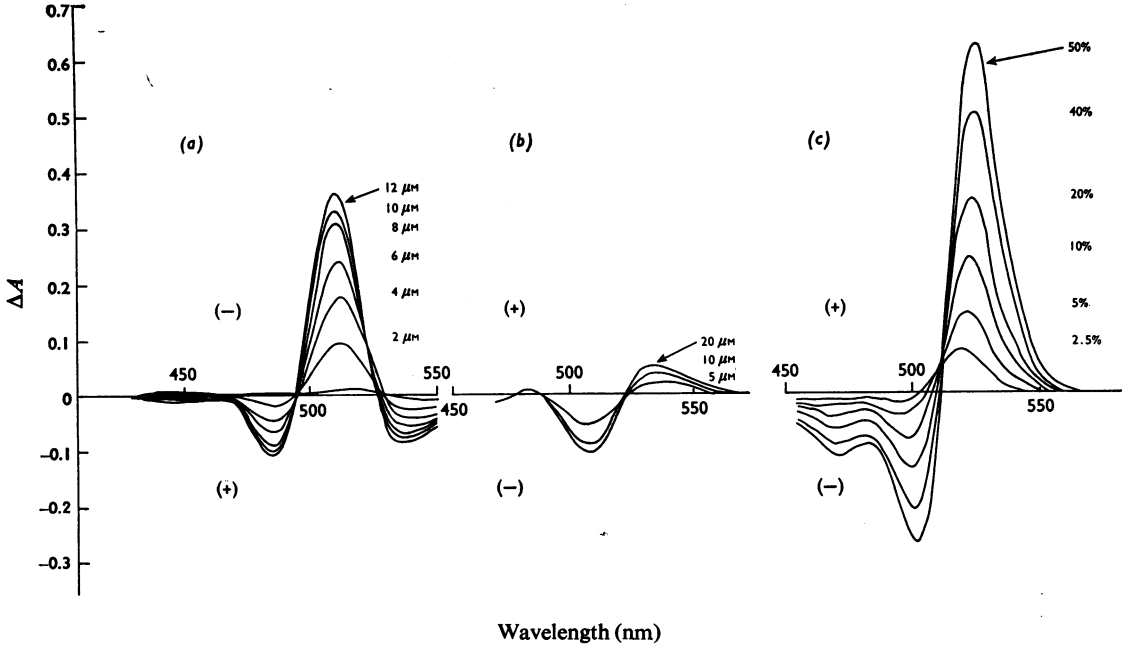

Fig. 2. *Difference spectra of the system mercurochrome–Na_2S (a), mercurochrome–cysteine (b) and mercurochrome–acetone (c) versus mercurochrome*

(*a*) Difference spectra of mercurochrome (20μM) in 0.1M-sodium carbonate/bicarbonate buffer, pH9.2, after the addition of Na_2S at final concentrations 2, 4, 6, 8, 10 or 12μM versus mercurochrome (20μM). (*b*) Difference spectra of mercurochrome (20μM) in 0.1M-sodium carbonate/bicarbonate buffer, pH9.2, after the addition of cysteine at final concentrations 5, 10 and 20μM versus mercurochrome (20μM). (*c*) Difference spectra of mercurochrome (20μM) in acetone/0.1M-sodium carbonate/bicarbonate buffer, pH9.2, mixtures containing 2.5, 5, 10, 20, 40 or 60% acetone versus mercurochrome (20μM). Spectra were recorded 15min after the mixing of the reactants; equilibrium of the systems was reached in less than 1min.

compounds. Fig. 2(*c*) shows difference spectra of the fluorochrome obtained in solutions of decreasing polarity. The difference spectra of the system mercurochrome–Na_2S versus mercurochrome (Fig. 2*a*) exhibit a negative absorption maximum at 510nm and two positive maxima at 485 and 535nm, whereas the maxima of the system mercurochrome–acetone versus mercurochrome (Fig. 2*c*) are a positive at 526nm and two negative ones at 502 and 470nm respectively. The spectral changes observed in the mercurochrome–cysteine system were less profound, exhibiting positive and negative maxima at 530 and 508nm respectively (Fig. 2*b*).

The possibility of mercurochrome combination with aromatic amino acids was examined during the titration of mercurochrome solutions (20μM) with the amino acids tryptophan, tyrosine, histidine and arginine up to final concentrations of 1.0mM. Minor or no spectral changes were observed in all cases.

Inhibitory effect of mercurochrome on native, 2,3-dicarboxyethylated and other modified forms of aspartate transaminase

Most of the reported catalytic and physicochemical characteristics of aspartate transaminase have been studied originally on a modified form of enzyme, the *S*-2,3-dicarboxyethylated or 'maleate' form of aspartate transaminase (Jenkins *et al.*, 1959; Braunstein, 1964). Fortunately, the partial alkylation does not result in substantial alteration of important properties of the enzyme, including absorption spectra, kinetics of catalytic action, rotatory dispersion etc. (Braunstein, 1964). However, appearance of specificity in the behaviour of the enzyme towards particular ligands because of maleate conjugation, and vice versa, could occur. A report on this topic has appeared (Polyanovsky *et al.*, 1973*b*).

In a series of experiments, native and 2,3-dicarboxyethylated transaminase (2.5μM) were incubated in 0.1M-Tris/HCl buffer, pH8.0, with different quantities of mercurochrome, giving molar ratios of mercurochrome/enzyme of 1–10. The results obtained are shown in Figs. 3(*a*) and 3(*b*). It appears that the conjugation of cysteine-45 with maleate in the modified enzyme substantially protects its catalytic activity from the inhibitory action of mercurochrome.

Semi-logarithmic plots of residual activity against time were obtained (Milhausen & Levy, 1975) by using the equation log[activity (% of initial)] = log[100 (%)]$-k't/2.303$. All plots were linear, indicating that the reaction was first-order with respect to enzyme concentration. For 2,3-dicarboxyethylated transaminase, the rate of inactivation was directly proportional to mercurochrome concentration, showing that the inactivation was also first-order with respect to mercurochrome. Thus a plot of the values of the pseudo-first-order rate constants, determined from the data of Fig. 4, against mercurochrome concentration gave a straight line with a slope corresponding to a second-order rate constant of $6.1\times10^2M^{-1}\cdot min^{-1}$. In contrast, with transaminase the time-course of inactivation was biphasic, and the overall reaction did not follow second-order kinetics. The value of the second-order rate constant of the first step of the reaction calculated from the pseudo-first-order rate constants was $1.62\times10^4M^{-1}\cdot min^{-1}$. The pseudo-first-order rate constant of the second step of the biphasic inactivation of transaminase, calculated by the same procedure, gave the approximate value $2.15\times10^3M^{-1}\cdot min^{-1}$ (Figs. 4*a* and 4*b*) for the second-order rate constant.

In contrast with mercurochrome, iodoacetate and 5,5′-dithiobis-(2-nitrobenzoate) interacted similarly

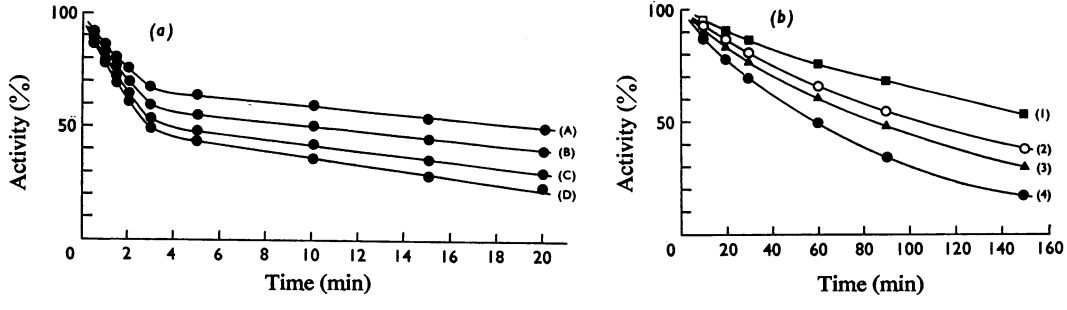

Fig. 3. *Effect of mercurochrome concentration on the rate of inactivation of transaminase (a) and the 2,3-dicarboxyethylated enzyme (b)*

Samples of transaminase (2.5μM) were incubated with various concentrations of mercurochrome (7.5, 10, 12.5 and 15μM) in 0.1M-Tris/HCl buffer, pH8.0 at 20°C. Samples were assayed at the indicated times. Curves (A), (B), (C), (D) and (1), (2), (3), (4) refer to native and modified enzymes for 7.5μM-, 10μM-, 12.5μM- and 15μM-mercurochrome respectively.

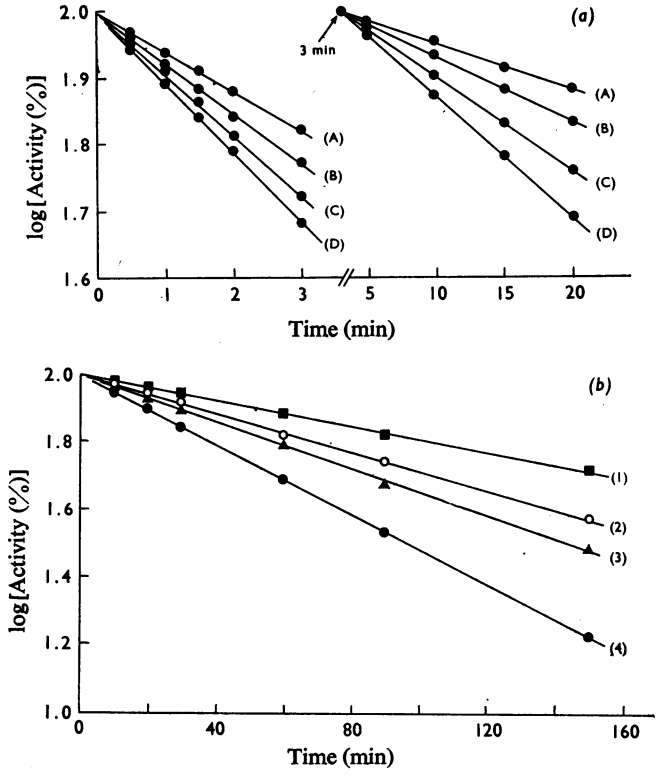

Fig. 4. *Semi-logarithmic plots of activity of transaminase (a) and the 2,3-dicarboxyethylated enzyme (b) with time at various mercurochrome concentrations*

The data from Fig. 3 were plotted to calculate the pseudo-first-order constants of the inactivation reaction. In the second step of the biphasic reaction in Fig. 3(*a*), the activity of every system at 3 min was taken as 100% activity for the calculation of the pseudo-first-order rate constants.

with native transaminase (Christen & Riordan, 1970; Riordan & Scandurra, 1975) and the 2,3-dicarboxyethylated enzyme without any major effect on the catalytic activity, demonstrating that the native and modified enzymes are very similar. A comparison of the effects of iodoacetate, 5,5′-dithiobis-(2-nitrobenzoate) and mercurochrome on the catalytic activities of native or modified enzyme is shown in Table 1, which also includes the catalytic activities of transaminase, the 2,3-dicarboxyethylated enzyme modified with iodoacetate or 5,5′-dithiobis-(2-nitrobenzoate) at the exposed thiol groups, and subsequently interacted with mercurochrome, in comparison with the titratable thiol groups of the various forms of the enzyme after enzyme–inhibitor interaction.

The above findings suggest that mercurochrome inactivates the modified enzyme either by combination with a thiol group not available to the usual thiol-group reagents or through its adsorption to a hydrophobic pocket of the enzyme complementary to the structure of mercurochrome. To test this hypothesis thiol groups on residues 45 and 82 were modified with *N*-ethylmaleimide and that on residue 390 was modified syncatalytically by an additional quantity of *N*-ethylmaleimide in a 2 ml reaction system containing 0.1 mM-transaminase, 70 mM-glutamate, 2 mM-2-oxoglutarate, 1 mM-pyridoxal phosphate and 1 mM-pyridoxamine phosphate in 0.05 M-Tris/HCl buffer, pH 8.0, for 24 h (Birchmeier *et al.*, 1973). At the end of this treatment, the reaction mixture was dialysed for 48 h against 0.1 M-Tris/HCl buffer, pH 8.0, changed frequently. The modified enzyme obtained under these conditions retained 7% of its original activity, but it was rather difficult to test mercurochrome binding to the essential thiol group of residue 390 only by the effect on enzyme inactivation, although the catalytic activity of the mercurochrome-treated

Table 1. *Effect of mercurochrome on the catalytic activity of native and modified transaminases compared with the content of titratable thiol groups after enzyme–inhibitor interaction*

Native or modified enzymes (2.5 μM) were allowed to interact for 60 min with mercurochrome (10 μM) in 0.1 M-Tris/HCl buffer, pH 8.0, before determination of the catalytic activity. Thiol groups of the enzymes were determined as described by Ellman (1959) with 0.4 mM-5,5'-dithiobis-(2-nitrobenzoate) in 0.1 M-Tris/HCl buffer, pH 8.0. Thiol groups were blocked with *N*-ethylmaleimide or 5,5-dithiobis-(2-nitrobenzoate) as described by Birchmeier *et al.* (1973).

Transaminase	Modifier	Catalytic activity (%)	Residual thiol-group content/molecule
1. Native	None	100	3.8
2. Native	Maleate	100	2.2
3. Native	Iodoacetate	110	0.4
4. Native	5,5'-Dithiobis-(2-nitrobenzoate)	90	—
5. Native	*N*-Ethylmaleimide, syncatalytically	7	0.2
6. 2,3-Dicarboxyethylated	Iodoacetate or 5,5'dithiobis-(2-nitrobenzoate)	100	0.2
7. Native	Mercurochrome	25	0.8
8. 2,3-Dicarboxyethylated	Mercurochrome	55	0.5
9. Carboxymethylated	Mercurochrome	50	0.2
10. 2,3-Dicarboxyethylated and carboxymethylated	Mercurochrome	60	0.3
11. Thionitrobenzoylated	Mercurochrome	65	0.3
12. 2,3-Dicarboxyethylated and thionitrobenzoylated	Mercurochrome	60	0.2
13. Syncatalytically modified with *N*-ethylmaleimide	Mercurochrome	2	—

Table 2. *Effect of single substrates or substrate pairs on the inhibitory action of mercurochrome on transaminase or the 2,3-dicarboxyethylated enzyme*

Native or modified enzyme (2.5 μM) was incubated with 70 mM-amino acid substrate or 2 mM-keto acid substrate, or with the same concentrations of substrate pairs in different combinations, and mercurochrome (10 μM) before determination of the catalytic activity. Incubation time was 30 and 90 min for native and modified enzymes respectively.

Substrate added	Residual activity (%)	
	Native	Modified
None	35	55
Aspartate	45	60
Glutamate	35	55
2-Oxoglutarate	50	65
Oxaloacetate	45	60
Aspartate + 2-oxoglutarate	50	65
Glutamate + 2-oxoglutarate	40	55
Aspartate + oxaloacetate	55	65
Glutamate + oxaloacetate	50	65
Aspartate + glutamate	40	55
Oxaloacetate + 2-oxoglutarate	65	60

enzyme was decreased from 7 to 2% of the activity of the native enzyme (Table 1). Further information about the type of complexes produced during the interaction between mercurochrome and syncatalytically modified enzyme is presented in Table 3.

Inhibitory effect of mercurochrome on native and 2,3-dicarboxyethylated aspartate transaminase in the presence of substrates

The reactivity of a thiol group depends not only on its state in any particular conformation of the enzyme molecule but also on the nature of the thiol-group reagents used. It was therefore decided to investigate the effect of substrates on the reactivity of the thiol groups of transaminase with a fast-reacting amphiphilic reagent such as mercurochrome.

In a series of experiments, transaminase or the 2,3-dicarboxyethylated enzyme was incubated with mercurochrome and a single substrate or a pair of an amino acid substrate and a keto acid substrate under a variety of combinations shown in Table 2. Transaminase interaction with the inhibitor took place in a 1 ml system, containing 2.5 μM-enzyme, 70 mM-amino acid substrate, 2 mM-keto acid substrate and various quantities of mercurochrome giving molar ratios of mercurochrome/enzyme of 1–10 in 50 mM-Tris/HCl buffer, pH 8.0. Minor changes in the inhibitory effect of mercurochrome, in any of the forms of transaminase used, were observed because of the presence of substrates. Slight protection of the catalytic activity of the enzyme by the substrates against mercurochrome inactivation was revealed. The protection was higher when 2-oxoglutarate was alone in the reaction mixture and lower when the system reacted with glutamate (Table 2).

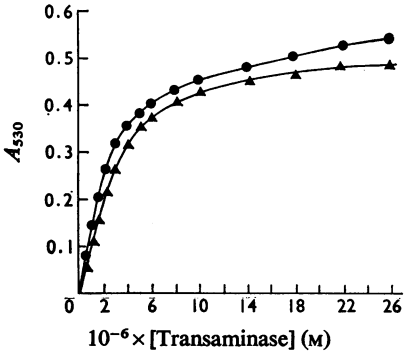

Fig. 5. *Spectroscopic changes of mercurochrome during its interaction with native and modified enzyme*
Changes in the A_{530} during the titration of mercurochrome solutions (20 μM) with increasing quantities of transaminase (●) or the 2,3-dicarboxyethylated enzyme (▲) in 0.1 M-sodium carbonate/bicarbonate buffer, pH9.2, are presented.

Changes in the absorption and emission spectra of mercurochrome during interaction with native or modified transaminase

The inhibitory action of mercurochrome on native or modified enzyme can also be followed spectroscopically. Studies were carried out by adding increasing amounts of native or modified transaminase ranging from 1.0 to 40 μM in 20 μM-mercurochrome solution in 0.1 M-sodium carbonate/bicarbonate buffer, pH9.2. Substantial spectroscopic changes were recorded. The characteristic absorption maximum of mercurochrome spectrum at 510nm gradually decreases during the titration with either form of transaminase, and a new absorption maximum appears at 520nm, representing a red shift in the spectrum maximum of the fluorochrome of the order of 10nm. Fig. 5 shows the effect of transaminase or the 2,3-dicarboxyethylated enzyme on the absorbance of mercurochrome at 530nm.

Interaction between mercurochrome and trans-

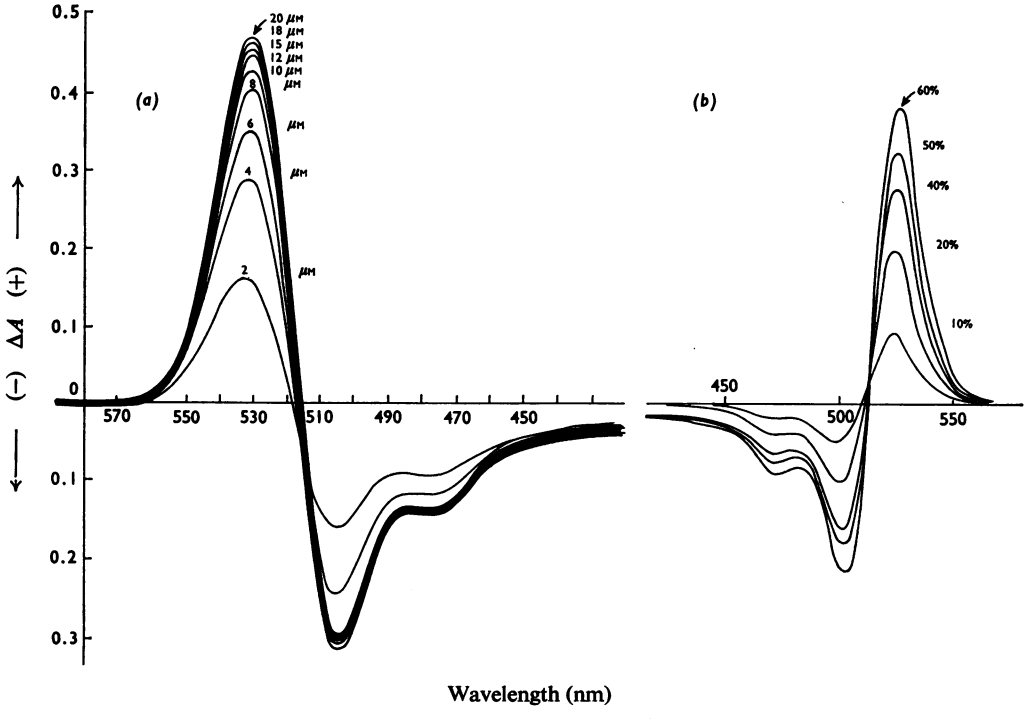

Fig. 6. *Difference spectra of mercurochrome–transaminase (a) or mercurochrome–ethanol (b) systems versus mercurochrome*
(*a*) Difference spectra of mercurochrome (20 μM) in 0.1 M-Tris/HCl buffer, pH8.0, after the addition of transaminase (final concentrations 2, 4, 6, 8, 10, 12, 15, 18 and 20 μM) versus mercurochrome (20 μM). (*b*) Difference spectra of mercurochrome (20 μM) in 0.1 M-Tris/HCl buffer (pH8.0)/ethanol mixtures containing 10, 20, 40, 50 and 60% ethanol versus mercurochrome (20 μM).

aminase is accompanied by a red shift in the absorption maximum of the fluorochrome. Difference spectra of mercurochrome solutions (20 μM) in 0.1 M-Tris/HCl buffer, pH 8.0, during the addition of increasing quantities of native transaminase, compared with analogous spectra of mercurochrome in ethanol/water mixtures of different composition, revealed that the spectral shifts recorded during mercurochrome–transaminase interaction were similar to those for mercurochrome dissolved in non-polar solvents. Fig. 6 shows that the difference spectra of the system mercurochrome–transaminase versus mercurochrome exhibit a positive maximum at 530 nm and a negative one at 504 nm, whereas the positive and negative maxima for the system mercurochrome–ethanol versus mercurochrome are 526 and 502 nm respectively. Spectra were recorded 15 min after the addition of transaminase to mercurochrome solutions. Most of the spectroscopic changes (more than 90%) take place during the first minute after the addition of the enzyme to the solution of the ligand. No spectroscopic changes were observed during the titration of solutions of dibromofluorescein with transaminase.

Spectroscopic studies were also carried out by adding mercurochrome (2–20 μM) in solutions of native or modified transaminase (50 μM). No major changes in the characteristic absorption maxima of the enzyme at 362 or 426 nm were observed. Spectral analysis of the products of reaction between mercurochrome and native or modified transaminases, exhibiting residual activity of approx. 5–20%, demonstrated that the enzyme derivatives were interconvertible between the forms with maxima at 362 and 333 nm, indicating partial activity of the complexes.

To explore further the previous findings, the fluorescence properties of mercurochrome were used. In a series of mercurochrome solutions (1.0 μM) in 0.1 M-sodium carbonate/bicarbonate buffer, pH 9.2,

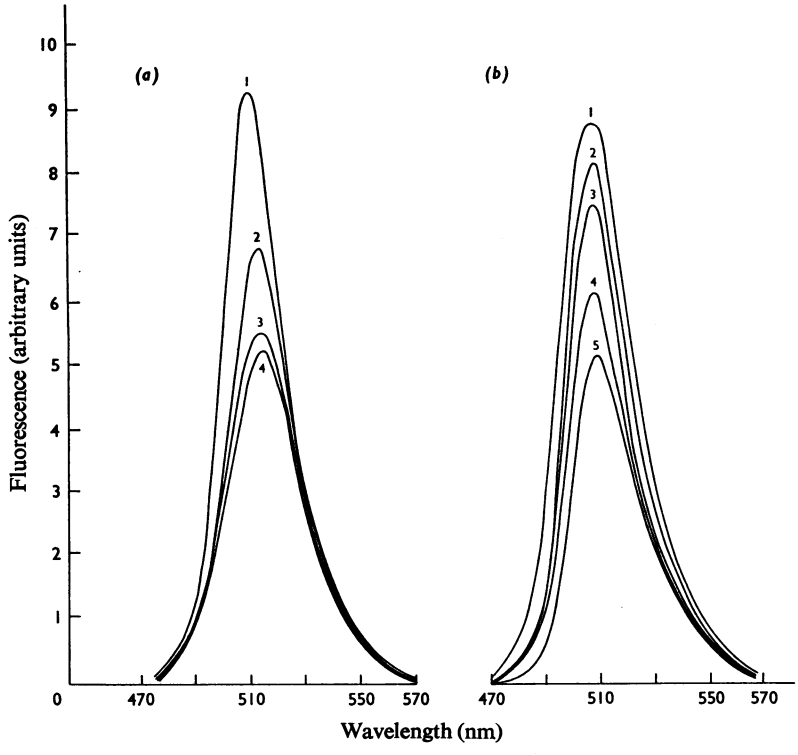

Fig. 7. *Changes in the emission spectra of the system mercurochrome–transaminase (a) and mercurochrome–cysteine (b)* (*a*) Emission spectra of mercurochrome (1.0 μM) before (1) and after the addition of transaminase in final concentrations 0.25 μM (2) 0.5 μM (3) or 1.0 μM (4). (*b*) Emission spectra of mercurochrome (1.0 μM) before (1) and after the addition of cysteine in final concentrations 0.125 μM (2), 0.25 μM (3), 0.5 μM (4) or 1.0 μM (5). Carbonate/bicarbonate buffer (0.1 M), pH 9.2, was used. Spectra are uncorrected; excitation was at 508 nm; other coefficients were slit sensitivity 0.1, sensitivity 35, multiplier 0.03.

transaminase was added at concentrations ranging from 0.1 to 1.0 μM. The results obtained are shown in Fig. 7, which also shows the recorded changes in the emission spectra of mercurochrome during the addition of increasing quantities of cysteine.

Isolation of complexes of transaminase or the 2,3-dicarboxyethylated enzyme with mercurochrome

Complexes of mercurochrome with native or modified transaminase were isolated as follows. In a final volume of 2ml of 0.1M-Tris/HCl buffer, pH8.0, transaminase (0.1mM) and mercurochrome (1.0mM) were allowed to interact for 1h at room temperature (20°C) and then for 24h in the cold-room (4°C). The excess of mercurochrome after the interaction was removed by passing the reaction mixture through a column (1cm×25cm) of Sephadex G-25 (fine grade) equilibrated and eluted with the above buffer. Eluate fractions (3ml) obtained with a fraction collector were used to determine the concentration of the protein and the fluorochrome by measuring the absorbance of the fractions at the absorption maxima of protein, fluorochrome and the complex (280, 510 and 520nm respectively). The actual concentrations of the enzyme and the fluorochrome in the complexes were determined by using the formula:

$$\text{Mercurochrome/transaminase (molar ratio)} = 2.1/[k(A_{280}/A_{520})-n],$$

taking into account that ε_{510} (mercurochrome) = 62000, ε_{280} (transaminase) = 130000, k (mercurochrome) = $A_{520}/A_{510} = 0.860$, n (mercurochrome) = $A_{280}/A_{510} = 0.180$. Calculations from triplicate experiments performed as described above gave molar ratios of mercurochrome/enzyme for the complexes shown in Table 3, which also shows the molar ratios for the complexes of mercurochrome with transaminase or with the 2,3-dicarboxyethylated enzyme treated previously with 5,5′-dithiobis-(2-nitrobenzoate) and iodoacetate and the complex of mercurochrome with transaminase modified syncatalytically by *N*-ethylmaleimide.

To test whether or not mercurochrome can conjugate with all the thiol groups of transaminase, native and modified enzymes in concentrations described above were interacted for 24h with mercurochrome in 0.1M-Tris/HCl buffer, pH8.0, containing 8.0M-urea. The excess mercurochrome after the interaction was separated by using Sephadex G-25 (fine grade) columns (1cm×25cm) equilibrated and eluted with 0.1M-Tris/HCl buffer, pH8.0, containing 8.0M-urea. The urea eluates containing the enzyme from either system were pooled and dialysed for 48h in the cold-room against 0.1M-Tris/HCl buffer, pH8.0, changed frequently. The molar ratio mercurochrome/enzyme of the dialysed systems determined as described above gave the values in Table 3.

The spectral characteristics of the isolated complexes of native and modified transaminases were similar to those observed previously during the spectroscopic titrations. Both complexes exhibited a red shift of about 10nm, compared with the spectrum of mercurochrome, with a new absorption maximum at 520nm.

To explore further the nature of the complexes between mercurochrome and native or modified enzyme, we have attempted to isolate and characterize mercurochrome-labelled peptides, using tryptic and chymotryptic hydrolysates of the complexes. Unfor-

Table 3. *Molar ratio of reactants in mercurochrome–transaminase complexes*

Stable complexes between mercurochrome and native or modified enzymes with the molar ratios shown in the Table were obtained by passing a reaction mixture consisting of 0.1mM-enzyme and 1mM-mercurochrome in 0.1M-Tris/HCl buffer, pH8.0, through a Sephadex G-25 (fine grade) column (1cm×25cm) equilibrated and eluted with the above Tris/HCl buffer.

Modifier used for modification before addition of mercurochrome	Native or modified enzyme utilized	Mercurochrome/enzyme molar ratio
None	Native	3.9
Maleate	2,3-Dicarboxyethylated	2.5
Iodoacetate	Carboxymethylated	1.8
Maleate and iodoacetate	2,3-Dicarboxyethylated and carboxymethylated	1.9
5,5′-Dithiobis-(2-nitrobenzoate)	Thionitrobenzoylated	1.5
Maleate and 5,5′-dithiobis-(2-nitrobenzoate)	2,3-Dicarboxyethylated and thionitrobenzoylated	2.1
N-Ethylmaleimide	Syncatalytically modified by *N*-ethylmaleimide	0.5
Urea (8M)	Native	5.3
Urea (8M)	2,3-Dicarboxyethylated	3.7

tunately the experiments were unsuccessful because the complex decomposed under the conditions of Dowex column chromatography and high-voltage electrophoresis.

The observed changes in the behaviour of transaminase after its interaction with mercurochrome should be considered as irreversible, since the inhibitor remains bound to the enzyme after gel filtration. In this case the dissociation constant of the inhibitor–transaminase complex could not be calculated from the observed spectroscopic changes. Irreversibility, however, is not easy to define in absolute terms (Gutfreund, 1965); dissociation of transaminase–mercurochrome complexes to their constituents can be accomplished by passing solutions of the complexes in 0.1 M-2-mercaptoethanol through Sephadex G-25 columns (1 cm × 25 cm) equilibrated and eluted with 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-2-mercaptoethanol.

Discussion

We discuss below some aspects of the interaction between mercurochrome and native or modified transaminases. Mercurochrome was chosen for its characteristic reactivities and spectroscopic properties in the visible region, an area of the spectrum where neither apoenzyme nor coenzyme or substrates absorb or emit (Figs. 1 and 2).

The native enzyme differs from the modified enzyme only with respect to the state of cysteine-45. It appears therefore that the conjugation of this thiol group with maleate results in changes in the physical and chemical state of the protein molecule expressed by the behaviour of the modified enzyme towards mercurochrome (Figs. 3 and 4). All modified enzymes were inhibited by mercurochrome (Table 1); since in carboxymethylated transaminase or 2,3-dicarboxyethylated enzyme two thiol groups per subunit of transaminase were modified by covalent binding of iodoacetate or iodoacetate and maleate respectively at cysteine residues 45 and 82, it seems that inactivation of transaminase by mercurochrome is not due to the conjugation of the inhibitor with those thiol groups.

Mercurochrome inactivates native or modified transaminases in the presence and in the absence of single substrates or different combinations of substrate pairs (Table 2). It seems that the syncatalytic inactivation phenomenon observed previously (Christen & Riordan, 1970; Riordan & Scandurra, 1975) does not apply to mercurochrome. This is possibly due to the fact that mercurochrome is unable to conjugate with the thiol group of residue 390 for steric reasons, or it might be due to the greater reactivity of the fluorescein derivative towards the enzyme.

The complexes between mercurochrome and native or differently modified transaminases demonstrated that mercurochrome conjugates with four thiol groups per molecule (two per subunit) of native enzyme but only with two thiol groups per molecule (one per subunit) of the modified enzyme (Table 3). It is concluded therefore that mercurochrome can combine with one more cysteine residue per subunit in native than in modified transaminase. This residue is probably cysteine-45, which is free in native transaminase but not in the 2,3-dicarboxyethylated enzyme. The complexes obtained with the enzyme modified at cysteine residues 45 and 82 (Table 3) resulted therefore either from combination of mercurochrome with one of the remaining thiol groups of transaminase (cysteine-191 or cysteine-252) or by adsorption of the inhibitor to an internal cavity of the enzyme complementary to the structure of mercurochrome. The former assumption does not explain why the native enzyme is not able to combine with six molecules of mercurochrome instead of four. One explanation could be that cysteine-82 is unable to combine with mercurochrome for structural reasons. This point is supported by the hydrophobicity of the mercurochrome-binding site on the enzyme and from the complexes between mercurochrome and transaminase obtained in the presence of 8 M-urea (Table 3). Even under these conditions the maximum numbers of inhibitor molecules that combined with native and modified enzymes were 3 and 2 per subunit respectively.

Spectroscopic titrations of mercurochrome with native or modified transaminase demonstrated quantitatively similar spectroscopic shifts (Fig. 5). This is possible because the second molecule of mercurochrome in the subunit, which is conjugated with the exposed cysteine-45 in native enzyme, has negligible effect on the red shift of the mercurochrome-absorption maximum (Fig. 2). It appears therefore from the spectral shifts that only one molecule of mercurochrome per subunit of native or modified enzyme contributes to the changes in the difference-spectra maxima at 530 nm (Figs. 5 and 6), or to the red shift in the absorption maximum of the inhibitor from 510 to 520 nm. These changes could originate from adsorption of the mercurochrome molecule on a hydrophobic site of transaminase, since similar spectroscopic shifts were induced in the mercurochrome absorption maximum by increasing concentrations of ethanol or acetone in water/ethanol or water/acetone mixtures (Figs. 2 and 6). The phenomena observed during mercurochrome–transaminase interaction suggest that a complex is formed very quickly with the enzyme which results in its inactivation, very possibly through the irreversible conjugation of the ligand with a thiol group.

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