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Effects of Metal Ion Substitution on Carboxypeptidase A Catalyzed Hydrolysis of *O-trans*-Cinnamoyl-L-β-phenyllactate†

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ABSTRACT: The effects of divalent metal ion substitution (Ni^{2+}, Co^{2+}, Cu^{2+}, and Hg^{2+}) for Zn^{2+} on the kinetics of carboxypeptidase A catalyzed hydrolysis of *O-trans*-cinnamoyl-L-β-phenyllactate have been determined in H_2O at 30 °C. The Cu(II) enzyme is inactive at pH values in the range 5.78–9.45, and the Hg(II) enzyme is inactive at pH 7.50 and 9.80. The Zn(II), Ni(II), and Co(II) enzymes give similar plots of k_{cat}, K_m, and k_{cat}/K_m vs. pH. The values of K_m are nearly pH independent in the range 5–9 but increase markedly at pH >9. The bell-shaped plots of k_{cat}/K_m vs. pH give pK_{app}^{E} values of 9.0, which are invariant, and near 6.0, which differ only slightly. The k_{cat} vs. pH profiles all show a sigmoidal region in which pK_{app}^{ES} values are closely similar [Zn(II), 6.2; Ni(II), 6.2; Co(II), 5.7] and a rapidly rising arm at pH >9. Values of k_{cat} on the upper arm indicate rate enhancements of 10^7–10^8 over nonenzymatic OH^--catalyzed hydrolysis of the ester. At lower pH (6–9), k_{cat}(lim) differs but slightly in the order Ni(II) > Co(II) > Zn(II). Modification of Glu-270 to the methoxyamide results in loss of activity at all pH values, showing that the carboxylate group is necessary for the reaction even at pH >9. The plot of k_{cat} vs. pH for hydrolysis of *O-trans*-cinnamoyl-L-mandelate is bell shaped. Values of k_{cat} are much less than those with *O-trans*-cinnamoyl-L-β-phenyllactate (230-fold at pH 8.20). These results indicate that (a) hydrolysis of an anhydride intermediate is rate determining at all pH values greater than 6 in hydrolysis of *O-trans*-cinnamoyl-L-β-phenyllactate, including pH >9, but possibly not in hydrolysis of *O-trans*-cinnamoyl-L-mandelate, (b) the apparent pK_a^{ES} at pH 6 is not that of metal ion bound water but represents either a composite constant or a change in the rate-determining step, and (c) both formation and breakdown of the anhydride intermediate must be greatly facilitated by the enzyme.

Carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC 3.4.12.2)[1] is a Zn(II)-requiring enzyme that catalyzes the hydrolysis of peptides and *O*-acyl derivatives of α-hydroxy carboxylic acids (Hartsuck & Lipscomb, 1971). Binding of esters requires the presence of metal ion whereas binding of peptides does not (Auld & Holmquist, 1974). However, the presence of metal ion is required for catalytic activity with both types of substrate. The primary amino acid sequence has been determined (Bradshaw et al., 1969), and the three-dimensional crystal structure has been elucidated by X-ray crystallographic analysis at 2-Å resolution (Ludwig & Lipscomb, 1973; Lipscomb, 1970). The X-ray studies have shown the zinc ion to be chelated to the carbonyl oxygen of poor peptide substrates. The carboxyl group of glutamic acid-270 has also been implicated in the catalytic process, and mechanisms have been suggested involving nucleophilic attack and classical general-base catalysis [proton transfer in the transition state from a water molecule (Lipscomb, 1970)].

Much of what is known of the effects of the metal ion in carboxypeptidase A reactions has been obtained from metal ion substitution studies (Hartsuck & Lipscomb, 1971; Ludwig & Lipscomb, 1973). Replacement of Zn(II) in the active site by a large series of metal ions has been achieved (Vallee et al., 1958; Coleman & Vallee, 1960, 1961; Davies et al., 1968; Auld & Vallee, 1970a). The configuration of the Ni(II) enzyme has been shown to be octahedral (Rosenberg et al., 1975b), whereas the coordination geometries of cobalt and copper carboxypeptidase were determined to be five-coordinate and tetrahedral, respectively (Rosenberg et al., 1973, 1975a). From the X-ray work, it is known that carboxypeptidase-bound zinc exhibits a distorted tetrahedral geometry (Lipscomb, 1970). These metalloenzymes all apparently show at least some esterase activity toward *O*-hippuryl-DL-β-phenyllactate except the Cu(II) (Coleman & Vallee, 1961) and the Co(III) enzyme (Van Wart & Vallee, 1978). The studies of the effects of metal ion substitution have been carried out with various substrates and different reaction conditions so that direct comparisons are difficult.

A variety of k_{cat} vs. pH profiles have been observed for hydrolysis of ester substrates of carboxypeptidase A (Hall et al., 1969; Carson & Kaiser, 1966; Bunting et al., 1974). As

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[1] Abbreviations: CPA, bovine pancreatic carboxypeptidase A; CPL, *O-trans*-cinnamoyl-L-β-phenyllactate; CM, *O-trans*-cinnamoyl-L-mandelate; CoCPA, Co^{2+}-substituted carboxypeptidase A; NiCPA, Ni^{2+}-substituted carboxypeptidase A; ΔH_i, enthalpy of ionization; Tris, tris(hydroxymethyl)aminomethane.

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a consequence, none of the apparent pK_a values that have been found to control K_m and k_{cat} have been conclusively assigned to the ionizable groups known to be present in the active site. The Zn(II) CPA catalyzed hydrolysis of the ester *O-trans*-cinnamoyl-L-β-phenyllactate (CPL) is characterized by a k_{cat} vs. pH profile showing a sigmoidal pH dependence at pH <9 with pK_{app}^{ES} = 6.2 (Hall et al., 1969). The profile has an ascending arm at pH values greater than 9, which perhaps indicates OH^- catalysis. Makinen et al. (1979) have recently investigated the reactions of the Zn(II) and Co(II) enzymes with (*p*-chlorocinnamoyl)-L-β-phenyllactate under cryogenic conditions, i.e., low temperature and an ethylene glycol–methanol–H_2O solvent system. They suggested that the apparent pK_a at ~pH 6 represents ionization of metal ion bound water. They did not carry the rate measurements to high enough pH to encounter the increasing rate with increasing pH that had been previously reported in reactions of CPL (Hall et al., 1969). If the pK_a of the metal ion bound H_2O is in fact as low as 6, then the increasing rate constants at pH >9 would be difficult to explain; they could not then be reflecting a reaction of metal ion bound OH^- since such a reaction will become pH independent at the pK_a. Greater understanding of the nature of the apparent pK_a^{ES} near 6 and of the apparent OH^--catalyzed reaction at pH >9 observed with CPL is consequently necessary for elucidation of the mechanism of action of the enzyme. Clearly, it is important to determine complete k_{cat} vs. pH profiles for hydrolysis of a specific ester substrate with a series of metalloenzymes in H_2O at ambient temperature, so that the reactions at all pH values, including pH >9, can be understood. If the pK_{app}^{ES} of 6 is that of metal ion bound water, then the pK_{app} should vary significantly with the metal ion, whereas this might not be the case for ionization of a functional group in the active site. In this respect, the Ni(II) enzyme would be especially informative since the pK_a of Ni(II)-bound H_2O is high (Basolo & Pearson, 1967); e.g., the pK_a of the hexaaquo complex of Ni^{2+} is 10.6, as compared to 8.8 and 8.9 for Zn^{2+} and Co^{2+}, and the configuration of the metal ion in NiCPA is octahedral (Rosenberg et al., 1975b).

In nonenzymatic ester hydrolysis, metal ion promoted OH^--catalyzed reactions have been found that proceed with extremely large rate enhancements, ranging from 10^4 to 10^9, depending on the metal ion (Wells & Bruice, 1977; Hay & Clark, 1977; Fife et al., 1979). These reactions give plots of log k_{obsd} vs. pH that are linear with slopes of 1.0 below the pK_a of metal ion bound water and that show considerable variation in the rate constants with the different metal ions (Fife et al., 1979; Fife & Squillacote, 1978). Therefore, in the search for possible correlations between the chemical studies of metal ion effects in nonenzymatic ester hydrolysis and reactions of carboxypeptidase A, we have studied the hydrolysis of *O-trans*-cinnamoyl-L-β-phenyllactate catalyzed by various metallo derivatives of carboxypeptidase A. Only the Zn(II) and Mn(II) enzymes have previously been employed in studies of the hydrolysis of CPL (Hall et al., 1969; Glovsky et al., 1972), and a pH–k_{cat} profile has only been obtained with the Zn(II) enzyme. This ester is a highly suitable substrate in that it does not lead to anomalous effects such as substrate inhibition or activation common with many other carboxypeptidase A substrates.

Experimental Procedures

Materials. Cinnamoyl chloride, mercury(II) chloride, L-β-phenyllactic acid, and L-mandelic acid were obtained from Aldrich Chemical Co. Sigma Chemical Co. supplied bovine carboxypeptidase A-DFP, *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's Reagent K), β-phenylpropionic acid, Trizma base, and ammediol. The aqueous chloride salts of zinc, cobalt, nickel, and copper were from E. Merck. Sephadex G-75 was obtained from Pharmacia Fine Chemicals, and methoxyamine hydrochloride was purchased from Fisher Chemical Co. The sodium salt of the ester *O*-hippuryl-L-phenyllactic acid was obtained from Vega Fox Biochemicals.

O-trans-Cinnamoyl-L-β-phenyllactate (CPL) was prepared by reaction of distilled cinnamoyl chloride with β-phenyllactic acid as described by Hall et al. (1969). The sodium salt of the substrate was made by careful titration of the acid with 0.10 N NaOH. The salt was then dissolved in hot acetone. The solution was evaporated to dryness, and the residue was triturated with dry diethyl ether. The UV spectrum and extinction coefficient of the synthesized compound were identical with those found by Hall et al. (1969).

O-trans-Cinnamoyl-L-mandelate (CM) and its sodium salt were prepared by the same methods as employed for the synthesis of *O-trans*-cinnamoyl-L-β-phenyllactate. The UV spectrum and extinction coefficient of the synthesized compound were identical with those found by Kaiser (1970) for cinnamoyl-DL-mandelate.

Carboxypeptidase A was prepared as previously described (Kaiser, 1970) with the following additions. After dialysis and centrifugation, the concentrated enzyme was applied to a 3.2 × 45 cm column of Sephadex G-75, equilibrated at pH 7.5 (0.05 M Tris-HCl buffer, μ = 0.5 M NaCl, with 0.2 mM Zn^{2+} added). The purified enzyme was concentrated on an Amicon Model 52 ultrafiltration unit to a final concentration of 0.2 mM. Sigma carboxypeptidase A lots 127C-8055 and 129C-8015 were used with identical results.

The various metallocarboxypeptidases were prepared by competitive replacement of Zn^{2+} by the desired metal ion. The chloride salts of Zn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Hg^{2+} were used to prepare the dialysis buffers. In all cases, 0.1 mM carboxypeptidase A was dialyzed against a 100-fold volume excess of a 5 mM metal ion solution in pH 7.5 (4 °C) 0.05 M Tris-HCl buffer with 1.0 M NaCl. The buffer was replaced every 12 h. The extent of metal ion replacement was determined by atomic absorption spectrophotometry. The metalloenzymes were stored in the presence of 5 mM metal ion.

Formation of Methoxyamide Carboxypeptidase. The method of modification of glutamic acid-270 in carboxypeptidase A by *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's Reagent K) and subsequent formation of the methoxyamide derivative was that of Petra (1971). Conditions were chosen to ensure maximum conversion of Glu-270 to the methoxyamine amide. A 60-μL aliquot of 120 mg/mL Woodward's Reagent K was added to 6 mL of 4×10^{-5} M enzyme (equilibrated at 25 °C) in pH 6.4 buffer (0.01 M phosphate with 1 M NaCl). A second identical aliquot was then added after 10 min. The reaction was stopped after 20 min with the addition of 1.5 mL of cold 8.9 M sodium formate (pH 7.0), and the solution was immediately placed in an ice bath. The modified enzyme was then separated from unreacted Woodward's Reagent K and its hydrolysis products by elution from a Sephadex G-25 column. The protein fraction was concentrated to a volume of 4 mL and added to 4 mL of 0.6 M methoxyamine hydrochloride at pH 6.4. This mixture was heated at 37 °C for 14 h. The solution was then centrifuged at 15 000 rpm at 4 °C for 15 min. The supernatant was chromatographed on the Sephadex G-25 column, and the protein peaks were pooled and dialyzed vs. 0.005 M phosphate–1 M NaCl buffer (pH 7.5) for 18 h at 4 °C. The enzyme modified by this method has been previously char-

acterized (Petra, 1971; Petra & Neurath, 1971).

Kinetic Measurements. The rates of nonenzymatic hydrolysis of *O-trans*-cinnamoyl-L-β-phenyllactate were monitored by following the disappearance of reactant at 290 nm at 30 °C with a Beckman Model 25 recording spectrophotometer. The ionic strength was maintained at 0.5 M with NaCl. The kinetic runs were initiated by introducing 20 μL of an acetonitrile ester stock solution (4 mM) into 2 mL of the appropriate NaOH solution. The rates of nonenzymatic hydrolysis of cinnamoyl-L-mandelate were monitored identically with those of CPL except that the ionic strength was maintained at 1.0 M with KCl and the kinetic runs were initiated by introducing 12–20 μL of an ester stock solution (3 mM) into 2 mL of the appropriate KOH solution. The pseudo-first-order rate constants for these reactions were calculated by using a linear-regression computer program.

The method of initial rates was used to monitor all enzymatic hydrolyses. The assay procedure was that of Hall et al. (1969) with the following modifications: assay volumes of 2 mL were used throughout the study, all deionized water and NaCl solutions were passed through a Chelex-100 column to ensure a minimal amount of endogenous metal ion, and all buffers had concentrations of 0.01 M and were maintained at an ionic strength of 0.5 M with NaCl. Below pH 7.0, assay mixtures for cobalt and nickel carboxypeptidase were supplemented with additional metal ion (10^{-4} M) (Latt & Vallee, 1971). Enzyme dilutions were made with 0.01 M Tris-HCl buffer with 1 mM added metal ion, except in the case of zinc carboxypeptidase, with which no excess zinc was added. Activity of the carboxypeptidase dilution was checked before and after data collection by following the disappearance of 0.4 mM *O*-hippuryl-L-phenyllactate at 254 nm. With CPL as a substrate, the enzyme concentration was 2–4 nM while substrate was varied from 0.05 to 2 mM. The rates of hydrolysis of CM were monitored identically with the method used for zinc carboxypeptidase A catalyzed hydrolysis of CPL. Extinction coefficients employed in the calculation of rate data were those of Kaiser (1970). The enzyme concentration in this case was 0.715 μM while substrate was varied from 0.5 to 10 mM. All rates were measured at 30 °C with a Pye Unicam SP8-100 recording spectrophotometer. Kinetic constants and standard deviations were calculated using a linear-regression computer program utilizing a V vs. $V/[S]$ plot of initial rate data. Measurements of pH were made on a Radiometer type PHM 22r pH meter at 4 or 30 °C. Protein concentrations were calculated from the extinction coefficient of carboxypeptidase A at 278 nm; $\epsilon = 6.42 \times 10^4$ M^{-1} cm^{-1} (Simpson et al., 1963). Concentrations of methoxyamide carboxypeptidase A were calculated from the extinction coefficient at 278 nm; $\epsilon = 7.8 \times 10^4$ M^{-1} cm^{-1} (Petra, 1971).

Results

Plots of $V/[E]$ vs. $V/[S]$ for carboxypeptidase A catalyzed hydrolysis of *O-trans*-cinnamoyl-L-β-phenyllactate were nicely linear at each pH, yielding values of k_{cat} and K_m. At least six substrate concentrations were employed in duplicate determinations at each pH and were chosen to bracket the apparent K_m. In Figure 1, the plots of k_{cat} vs. pH are presented for the Zn(II), Ni(II), and Co(II) enzymes. In all cases, the plots show a change of slope near pH 6. Values of pK_{app} and the pH-independent limiting rate constant [k_{cat}(lim)] for the reaction at pH > pK_{app} are given in Table I. At pH >9, k_{cat} increases with increasing pH. Values of K_m are nearly pH independent in the pH range 5–9 but increase sharply at pH >9, as seen in Figure 2 where K_m is plotted vs. pH. The K_m constants are quite similar for the three metalloenzymes;

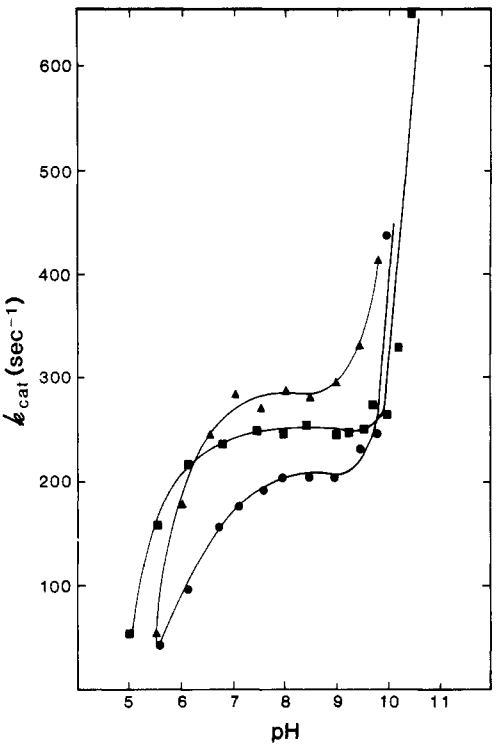


FIGURE 1: Plots of k_{cat} vs. pH for hydrolysis of *O-trans*-cinnamoyl-L-β-phenyllactate catalyzed by Zn(II) (●), Ni(II) (▲), and Co(II) (■) carboxypeptidase A at 30 °C and μ = 0.5 M. The enzyme concentration was (2–4) × 10^{-9} M, and substrate was varied from 0.05 to 2.0 mM.

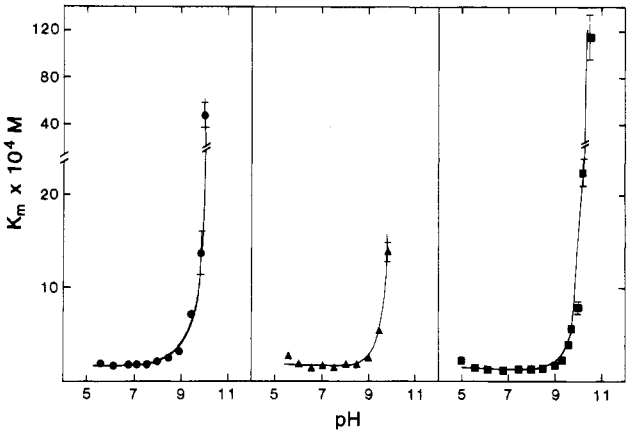


FIGURE 2: Plots of K_m vs. pH for hydrolysis of *O-trans*-cinnamoyl-L-β-phenyllactate catalyzed by Zn(II) (●), Ni(II) (▲), and Co(II) (■) carboxypeptidase A at 30 °C and μ = 0.5 M. The enzyme concentration was (2–4) × 10^{-9} M. Substrate was varied from 0.05 to 2.0 mM.

Table I: Kinetic Constants for Metallocarboxypeptidase A Catalyzed Hydrolysis of *O-trans*-Cinnamoyl-L-β-phenyllactate at 30 °C

enzyme	pK_1^{ES} [a]	pK_1^{E} [b]	pK_2^{E} [b]	k_{cat}(lim) (s^{-1})
ZnCPA	6.2 ± 0.1	6.3 ± 0.2	9.0 ± 0.2	195 ± 10
CoCPA	5.7 ± 0.1	5.8 ± 0.2	9.0 ± 0.2	240 ± 12
NiCPA	6.2 ± 0.1	6.4 ± 0.2	9.0 ± 0.2	295 ± 21

[a] Determined from plots of log k_{cat} vs. pH. [b] Determined from plots of log (k_{cat}/K_m) vs. pH.

K_m(lim), the pH-independent constant (pH 5–9), is 1.8×10^{-4} M, 1.4×10^{-4} M, and 1.2×10^{-4} M for the Zn(II), Ni(II), and Co(II) enzymes, respectively. Plots of log k_{cat}/K_m vs. pH are presented in Figure 3. The plots are in each case bell

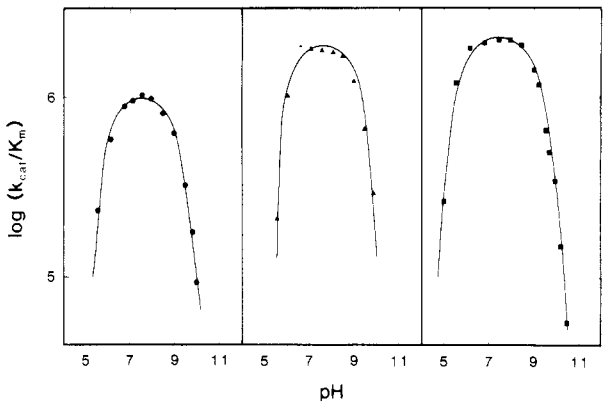


FIGURE 3: Plots of log (k_{cat}/K_m) vs. pH for hydrolysis of *O-trans*-cinnamoyl-L-β-phenyllactate catalyzed by Zn(II) (●), Ni(II) (▲), and Co(II) (■) carboxypeptidase A at 30 °C and μ = 0.5 M. The enzyme concentration was (2–4) × 10^{-9} M, and substrate was varied from 0.05 to 2.0 mM.

Table II: Glutamic Acid-270 Modification

sample	k_{cat} (s^{-1}) (pH 7.50)	% control	k_{cat} (s^{-1}) (pH 9.65)	% control
ZnCPA (native)	225	100	260	100
methoxyamide CPA[a]	18	8	24	9
methoxyamide CPA + inhibitor[b]	162	72	195	75

[a] The enzyme was treated with Woodward's Reagent K followed by conversion to the methoxyamide, employing the method of Petra (1971). [b] Enzyme preincubated with β-phenylpropionic acid was treated with Woodward's Reagent K and then methoxyamine, employing the method of Petra (1971).

shaped. The apparent pK_a values, pK_1 and pK_2, obtained from Figure 3 are given in Table I. The Cu(II) and Hg(II) enzymes were also prepared but were found to be inactive toward CPL, within the limits of the extent of metal ion substitution. The Cu(II) enzyme was found to be inactive at eight pH values in the range 5.78–9.45 at a substrate concentration of 5 × 10^{-4} M. There was slight residual activity of the Hg(II) enzyme toward CPL at pH 7.50 and 9.80, but it could be suppressed by additional dialysis vs. Hg^{2+}. However, the residual activity of the Hg(II) enzyme toward the substrate *O*-hippuryl-L-β-phenyllactate could not be eliminated by this treatment [see Davies et al. (1968)].

The carboxyl group of Glu-270 was selectively modified to the methoxyamide by the method of Petra (1971), employing *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's Reagent K) followed by treatment with methoxyamine. This modification produces an enzyme that is inactive toward CPL at both pH 7.50 and 9.65 as shown by the data in Table II. Prior incubation of the enzyme with the inhibitor β-phenylpropionic acid at a concentration of 4 mM protects the enzyme against inactivation by Woodward's Reagent K and methoxyamine, thereby showing that the active site is being modified.

In Figure 4, a plot is presented of k_{cat} vs. pH for hydrolysis of *O-trans*-cinnamoyl-L-mandelate at 30 °C catalyzed by Zn-CPA. Although error limits on some of the points are relatively large, the plot is clearly bell shaped. This ester has been studied previously (Kaiser, 1970), but a rate determination was made only at pH 7.5. Values of k_{cat} are much less than those with CPL. For example, at pH 8.2 where the rate is maximal, k_{cat} is 0.92 s^{-1} with the mandelate ester, in contrast with k_{cat} = 207 s^{-1} for the corresponding phenyllactate. The

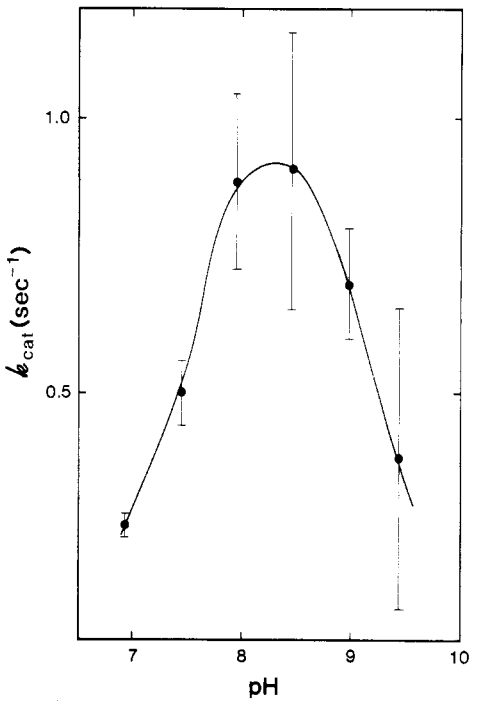


FIGURE 4: Plot of k_{cat} vs. pH for hydrolysis of *O-trans*-cinnamoyl-L-mandelate catalyzed by carboxypeptidase A at 30 °C and μ = 0.5 M. The enzyme concentration was 7.15 × 10^{-7} M, and substrate was varied from 0.5 to 10 mM. Error bars indicate standard deviation error limits.

values of K_m are larger with CM and show a gentle sigmoidal increase in the pH range 6.93–8.98 ranging from 1.80 × 10^{-3} M to 7.21 × 10^{-3} M. The value of k_{OH}, the second-order rate constant for nonenzymatic OH^--catalyzed hydrolysis of CM, is 0.024 M^{-1} s^{-1} at 30 °C, quite similar to k_{OH} for hydrolysis of CPL, 0.017 M^{-1} s^{-1}.

Discussion

The k_{cat} vs. pH profiles for carboxypeptidase A catalyzed hydrolysis of *O-trans*-cinnamoyl-L-β-phenyllactate can be considered to contain a bell-shaped or sigmoid region with a superimposed region at pH >9 in which the rate constants increase with increasing pH (Hall et al., 1969; Suh & Kaiser, 1976). Values of pK_1 are near 6 and are quite similar whether determined from plots of log k_{cat} or log (k_{cat}/K_m) vs. pH, as seen in Table I; i.e., pK_1^E and pK_1^{ES} are nearly identical. Comparable pK_{app} values have also been observed in peptide hydrolysis (Auld & Vallee, 1970b, 1971). The pK_a near 6 in the plots of log (k_{cat}/K_m) vs. pH has been assigned to Glu-270 (Hartsuck & Lipscomb, 1971; Suh & Kaiser, 1976; Auld & Vallee, 1970b; Kaiser & Kaiser, 1972), and this is supported by chemical modification of the enzyme. Modification of carboxypeptidase A with *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Petra, 1971) or *N*-(bromoacetyl)-*N*-methylphenylalanine (Hass & Neurath, 1971) proceeds with concomitant loss of activity toward esters and peptides, and the rate of inactivation of the enzyme shows dependence on the ionization of a group in the enzyme with a pK_a of approximately 7. However, the pK_1^{ES} for mandelate esters is >7 (Carson & Kaiser, 1966), and k_{cat} for hydrolysis of *O*-hippuryl-L-β-phenyllactate is pH independent in the pH range 5–10 (Bunting et al., 1974). Furthermore, the ΔH_i^{ES} of ~7 kcal/mol is not in accord with ionization of a carboxyl group (Makinen et al., 1979; Auld & Vallee, 1971); a ΔH_i of ~1.0 kcal/mol might be expected for ionization of a carboxyl group. Of course, the environment in the active site of the enzyme could be quite different than in aqueous solution, which could

result in a perturbed value of ΔH_i.

It has recently been suggested that the pK_{app}^{ES} in hydrolysis of (*p*-chlorocinnamoyl)-L-β-phenyllactate of 6.1 is the pK_a of a metal-bound water molecule (Makinen et al., 1979), although this pK_a is much lower than generally observed in the complexes of Zn^{2+} (the pK_a of the aquo complex is 8.8) (Basolo & Pearson, 1967). It was argued (Makinen et al., 1979) that a low pK_a might result from the tetrahedral coordination of Zn^{2+} and/or a hydrophobic environment. If the pK_1^{ES} were indeed the pK_a of metal ion bound water, then the value should vary considerably depending on the identity of the metal ion. Makinen et al. (1979) found that the pK_1^{ES} for the Co(II) enzyme was 4.9 in an ethylene glycol–methanol–water medium. It can be seen in Table I that, in water as the solvent, the pK_1 values for the Zn(II), Co(II), and Ni(II) enzymes in hydrolysis of CPL are reasonably similar. The pK_1^{ES} for the Co(II) enzyme (5.7) is lower than that of the Zn(II) or Ni(II) enzymes, but the difference is slight. The value of pK_1^{ES} for the Ni(II) enzyme (6.2) is quite significant since the pK_a of Ni^{2+}-bound water is usually quite high (10.6 in the hexaaquo complex; Basolo & Pearson, 1967). Since the configuration of Ni(II) in CPA is octahedral and appears to be five-coordinate in the β-phenylpropionic acid complex (Rosenberg et al., 1975b), the argument (Makinen et al., 1979) that a drop in the pK_a of metal ion bound water would occur due to a tetrahedral configuration cannot be applied to the nickel enzyme. Furthermore, the enzyme-catalyzed OH^- reaction at pH >9 is not in accord with ionization of metal ion bound water at low pH. Therefore, it is highly unlikely that the pK_1^{ES} is the pK_a of metal ion complexed water.

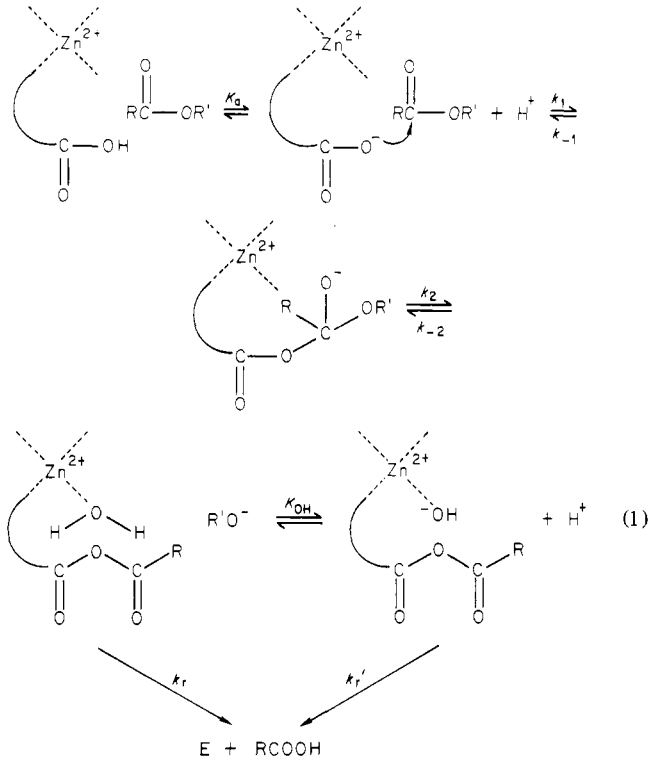
The pK_2^E of approximately 9 obtained in the hydrolysis of various substrates for carboxypeptidase A has been tentatively ascribed to ionization of the phenolic group of a tyrosine residue (Auld & Vallee, 1970b, 1971) or to ionization of the metal ion coordinated water molecule (Suh & Kaiser, 1976; Kaiser & Kaiser, 1972; Glovsky et al., 1972). Acetylation of Tyr-248 has no significant effect on $K_{m\,app}$ for hydrolysis of CPL and reduces k_{cat} by only a factor of 2 (Glovsky et al., 1972). The values of pK_2 in Table I for hydrolysis of CPL are constant for the Zn(II), Co(II), and Ni(II) enzymes. The pK_2^E for the Mn(II) enzyme (9.3) in hydrolysis of CPL is also quite similar (Glovsky et al., 1972). These pK_2^E values are of the magnitude expected for ionization of metal ion complexed water, but again, such a close similarity for the different metalloenzymes would not be anticipated. Values of pK_2^E close to 9 were also found for various metallocarboxypeptidases in hydrolysis of tripeptide substrates (Auld & Vallee, 1970b). Therefore, it is probable that pK_2^E reflects the ionization of another functional group in or near the active site. A pK_2^E of 9 has been observed with other ester substrates (Bunting & Chu, 1976), and it was suggested that the ionization may control an important conformational change affecting substrate binding and therefore may not occur in the active site region to which the substrate binds.

The k_{cat} vs. pH profiles in Figure 1 at pH >9 resemble those found in metal ion catalyzed ester and anhydride hydrolysis reactions in which metal ion promoted OH^- catalysis occurs (Wells & Bruice, 1977; Hay & Clark, 1977; Fife & Squillacote, 1978; Fife et al., 1979; Breslow et al., 1975). The second-order rate constants that can be calculated for the apparent hydroxide ion catalyzed reactions of CPL at pH >9 range from 10^6 to 10^7 M^{-1} s^{-1}, which are many orders of magnitude greater than the nonenzymatic second-order rate constant k_{OH} for OH^--catalyzed hydrolysis of CPL (0.017 M^{-1} s^{-1}). Therefore, the reactions are markedly catalyzed by the enzyme. If an internal attack of metal ion bound OH^- were occurring, the reactions would become pH independent at the pK_a. It is not experimentally feasible to continue rate measurements to the very high pH values required to establish the pK_a, but in view of the profiles at pH >9, the pK_2^{ES} values must necessarily be higher than 10.5. While such a high pK_a is reasonable for Ni(II), the values required for Zn(II)- and Co(II)-bound water would be somewhat higher than those observed in hexaaquo complexes. Since one of the metal ion liganding groups in carboxypeptidase A is a carboxylate ion (Hartsuck & Lipscomb, 1971; Lipscomb, 1970), which will reduce the positive charge on the metal ion, a relatively high pK_a for metal ion complexed water would not be surprising. Complexation of the metal ion with the substrate or anhydride intermediate could, of course, further decrease the positive charge and increase the pK_a of metal ion bound water in the ES complex.

The enhancements in the second-order rate constants k_{OH} at saturating concentrations of Ni^{2+} in nonenzymatic OH^--catalyzed hydrolysis of five esters with metal ion chelating functional groups and poor leaving groups similar in pK_a to phenyllactic acid are closely similar, ranging from 1.2×10^4 to 4.9×10^4 (av 2.5×10^4) (Wells & Bruice, 1977; Hay & Clark, 1977; Fife & Pyzystas, 1982). All of these esters allow the metal ion to bind very strongly to the reactant in excellent steric position to promote OH^- catalysis. This rate enhancement appears to be maximal in Ni^{2+} catalysis for esters with aliphatic alcohol leaving groups, although much larger rate enhancements can be achieved in the reactions of phenolic esters (Fife et al., 1979). Therefore, a facilitation in rate of 10^4 would also be expected to hold for carboxypeptidase A catalyzed reactions if metal ion promoted OH^--catalyzed hydrolysis of the ester substrate were occurring in reactions of the Ni(II)-substituted enzyme. However, in comparison with the k_{OH} for nonenzymatic hydrolysis of CPL at 30 °C (0.017 M^{-1} s^{-1}), the rate enhancement in the NiCPA-catalyzed reaction at pH >9 is 10^8. This strongly indicates that the enzymatic metal ion facilitated OH^- reaction at high pH involves a species with a good leaving group, i.e., the anhydride intermediate produced by nucleophilic attack of Glu-270 on the substrate. Recent cryogenic studies support a nucleophilic mechanism with ester substrates at pH 7.5 (Makinen et al., 1976).

Modification of the carboxyl group of Glu-270 by conversion to the methoxyamide inactivates carboxypeptidase A at the pH value of 6.4 toward hippuryl-DL-phenyllactate (Petra, 1971). Similar treatment of the enzyme in the present work abolishes activity toward CPL at *all* pH values studied, including pH >9. These results suggest that the Glu-270 carboxyl is essential for activity in all regions of the k_{cat} vs. pH profile and, consequently, support the viewpoint that the reaction at pH >9 involves a breakdown of the anhydride intermediate. Thus, it is probable that Glu-270 functions as a nucleophile in reactions of the ester at all pH values that are experimentally accessible.

The simplest explanation of the profiles for hydrolysis of *O-trans*-cinnamoyl-L-β-phenyllactate is that nucleophilic attack by Glu-270 occurs followed by rate-determining breakdown of the anhydride at all pH values greater than 6; i.e., the scheme followed is that of eq 1 or a kinetically equivalent scheme in which the metal ion complexes the anhydride intermediate. Kuo & Makinen (1982) have proposed that the configuration of the zinc ion is altered from tetracoordinate in the free enzyme to pentacoordinate in the anhydride intermediate. The metal ion could therefore complex both the



anhydride and a water molecule. Anhydride solvolysis would then be H_2O catalyzed below pH 9 and OH^- catalyzed at pH >9 with these reactions facilitated by the metal ion. The k_{cat} vs. pH profile for such reactions would be sigmoidal and would increase with increasing pH at pH >9, as observed. Kaiser & Kaiser (1972) previously suggested on the basis of the D_2O solvent isotope effect at pD 8.05 for hydrolysis of CPL ($k_{cat}^{H_2O}/k_{cat}^{D_2O}$ = 2) that breakdown of anhydride is rate limiting. Interpretation of solvent isotope effects in enzymatic reactions is difficult (Jencks, 1963), but the observed effect is consistent with rate-determining anhydride breakdown in the pH range where k_{cat} is pH independent. Also consistent are the changes in k_{cat} at pH 7.5 that occur upon variation of the para substituent of para-substituted cinnamoyl-L-β-phenyllactate esters (Kaiser et al., 1974), i.e., variation of the carboxylate portion of the ester with constant alcohol leaving group. The apparent pK_1^{ES} cannot easily be ascribed to either Glu-270 in view of the measured value of ΔH_i or ionization of metal ion bound water because of the present work. If it is considered that the ΔH_i is not consistent with ionization of a carboxyl group, then there remain only two possibilities that are consistent with all of the data. Either pK_1^{ES} is a composite constant, or it represents the pH at which a change in the rate-determining step occurs; i.e., pK_1^{ES} is not the pK_a of any single functional group.

Any preequilibrium step (tetrahedral-intermediate formation, etc.) occurring after the ionization step can lower the apparent pK_a if the equilibrium constant is sufficiently large. The effects of preequilibria on apparent pK_a values have previously been discussed in detail (Bruice & Schmir, 1959). Significant alterations in pK_{app} values have been observed in chemical intramolecular reactions due to a preequilibrium step. If one assumes saturation of the enzyme with ester and omits equilibria involving metal ion coordination, the scheme of eq 1 would yield eq 2 for k_{cat} at any pH, considering anhydride

$$k_{cat} = \frac{k_r K_a K_{eq} a_H + k_r' K_{OH} K_a K_{eq}}{a_H^2 + K_a a_H + K_a K_{eq} a_H + K_{OH} K_a K_{eq}} \quad (2)$$

formation to be an equilibrium step [$K_{eq} = k_1 k_2/(k_{-1}k_{-2})$]. Since the alcohol product binds strongly in the active site (K_i = 5.8 × 10^{-5} M at pH 7.5) (Hall et al., 1969), reversibility (k_{-2}) would reasonably be expected although it has not as yet been detected (Hall & Kaiser, 1967). If K_aK_{eq} is less than K_a, then k_{cat} at pH <9 will be given by eq 3 and the apparent

$$k_{cat} = \frac{k_r K_a K_{eq}}{K_a + a_H} \quad (3)$$

pK_a^{ES} at pH 6 will be the pK_a of the Glu-270 carboxyl group. On the other hand, if K_aK_{eq} is greater than K_a, then k_{cat} at pH <9 will be given by eq 4, so that k_{cat} at pH >6 is independent of the value of K_{eq} but the apparent pK_a^{ES} at pH 6 is a composite constant K_aK_{eq}. The scheme of eq 3 would require that k_{cat} in the pH range 6–9 would be k_rK_{eq}. If K_aK_{eq} < K_a, then the value of k_r would necessarily be greater than 10^3–10^4 s^{-1}, which is much larger than expected for water attack on an anhydride (Breslow et al., 1975; Fife & Przystas, 1983). In addition, in view of the different k_{cat} vs. pH profiles for ester substrates, eq 3 would require that the pK_a of the Glu-270 carboxyl group changes in the ES complex over an extremely wide range as the substrate is varied. For K_1 to be a composite constant, K_{eq} would be required to be unity or greater. This is quite reasonable for such an intracomplex reaction. Experiments designed to trap the anhydride intermediate with hydroxylamine have given negative results (Breslow et al., 1975). However, this could simply reflect the inability of hydroxylamine to compete with the rapid metal ion catalyzed hydrolysis reaction. The lack of detectable transesterification with labeled β-phenyllactate in reactions of CPL is in accord with a relatively slow reversal step [reaction of alcohol with anhydride (Hall & Kaiser, 1967)] but can also be explained by a slow rate of binding of labeled β-phenyllactate from solution in comparison with hydrolysis, reversibility only occurring via the alcohol generated in the anhydride formation step (Kaiser & Kaiser, 1972).

$$k_{cat} = \frac{k_r K_a K_{eq}}{K_a K_{eq} + a_H} \quad (4)$$

The other possibility for the apparent pK_a^{ES} near pH 6 in the hydrolysis of CPL is that a change in the rate-limiting step occurs at that pH. This could involve a change from rate-determining anhydride breakdown to rate-determining nucleophilic attack as the pH is lowered. A change in the rate-limiting step with changing pH might reasonably be expected since water attack on the anhydride intermediate would be pH independent,[2] whereas nucleophilic attack by the carboxylate anion of Glu-270 would be limited by the concentration of the ionized species at low pH. This explanation has the advantage of simplicity in that it does not demand reversibility and a large K_{eq}. The pH independence of K_m at pH less than 7 (Figure 2) in the pH range where k_{cat} is decreasing as the pH decreases is consistent with K_m equal to K_s, the dissociation constant of the ES complex, as would be the case if anhydride formation were rate determining at pH <6. The measured K_m would only equal K_s if $k_r > k_2$ or if K_{eq} were small (<1).

If pK_1^{ES} in carboxypeptidase A catalyzed ester hydrolysis is indeed a complex constant or a change in the rate-limiting step, then the different k_{cat} vs. pH profiles for ester substrates become understandable. The near pH independence of k_{cat} for *O*-hippuryl-L-β-phenyllactate in the pH range 5–10

[2] The hydrolysis of mixed cinnamic acid anhydrides in which there are additional metal ion chelating groups proceeds via both water- and OH^--catalyzed pathways in the presence of saturating concentrations of Zn^{2+}, Co^{2+}, or Ni^{2+} (Fife & Przystas, 1983).

(Bunting et al., 1974) would require a pK_{app} less than 5, which would result if K_{eq} were 10-fold larger than that with CPL. Likewise, the $pK_1^{ES} > 7$ for mandelate esters (Carson & Kaiser, 1966) could reflect a K_{eq} considerably less than that of CPL or a difference in the rate-determining step. A change in the rate-limiting step would also explain the various pK_1^{ES} values for the different esters because the pH at which such a change occurred would be dependent on the structure of the ester substrate. By excluding mechanistic possibilities in which pK_1^{ES} is assigned to a specific functional group (metal ion bound water or Glu-270), it is possible to correlate a large array of data on hydrolysis of the various ester substrates for the enzyme that are otherwise quite contradictory.

If breakdown of an anhydride intermediate were strictly rate limiting in the enzyme-catalyzed hydrolysis of esters with $K_aK_{eq} > K_a$, then variation of the alcohol portion of the substrate molecule with a constant carboxylic acid portion might not lead to a change in k_{cat} since the anhydride intermediate would not be altered. This would clearly be the case if the alcohol leaving group departed from the active site before the anhydride intermediate was hydrolyzed. In fact, the alcohol will be strongly bound and may not leave until the anhydride hydrolyzes. It would be expected, however, that the major effect of the presence of the alcohol in the active site would be on the reverse reaction, i.e., on k_{-2}. *O-trans*-Cinnamoyl-L-mandelate has k_{cat} values that are much less than those of CPL. The pK_{app} values are also considerably different with mandelate esters; pK_1^{ES} is approximately 7.5 with CM (see Figure 4) and 7.2 with the acetate ester (Carson & Kaiser, 1966). It is likely with mandelate esters that either $K_aK_{eq} < K_a$ or that nucleophilic attack by Glu-270 becomes rate limiting. The latter possibility would explain the differently shaped k_{cat}–pH profile for CM in the high-pH region as compared with CPL; at high pH values, the contribution of k_r' to anhydride breakdown gives rise to an apparent hydroxide ion catalyzed reaction in the hydrolysis of phenyllactate esters that would not then be observed in hydrolysis of CM. The large K_m for CM could reflect a poor steric fit of the ester with consequent difficulty of nucleophilic attack. In this regard, it is important to note that the K_m of CM has a different pH dependence than that of CPL but one that is similar to that of *O*-acetyl-L-mandelate (Carson & Kaiser, 1966).

Comparison with Nonenzymatic Ester Hydrolysis. The pH-independent reaction at pH 7–9 in the Ni(II)-substituted enzyme catalyzed hydrolysis of CPL has $k_{cat} = 295\ s^{-1}$ at 30 °C, and values of the rate constants for Zn- and CoCPA are similar. These limiting k_{cat} values for the enzyme reaction at pH <9 are extremely large, considering that the pK_a of the leaving group must be in the range 12–14. It is doubtful whether the steric fit of the carboxyl group of Glu-270 and the substrate ester carbonyl could be substantially improved over that of the ester 2-pyridylmethyl hydrogen phthalate (pK_a of the leaving group = 13.9), and yet, k_{cat}(lim) for the enzyme is at least 10^8 times larger than the pH-independent rate constant for carboxylate ion participation in hydrolysis of the phthalate ester (Fife & Pyzystas, 1980). Divalent metal ion catalysis has been observed in carboxylate nucleophilic reactions of esters having poor leaving groups (Fife & Przystas, 1980, 1982). In those reactions, the metal ion exerts its catalytic effect through a transition-state effect in which the leaving group is stabilized, but with rate enhancements of only 10^2. On the other hand, rate constants for nonenzymatic Ni^{2+}-, Co^{2+}-, and Zn^{2+}-catalyzed hydrolysis of the mixed anhydride of cinnamic acid and 2-carboxypicolinic acid are moderately similar to those in the CPA-catalyzed hydrolysis of CPL.[2] Rate-limiting anhydride hydrolysis in the enzyme reaction of CPL therefore suggests that the carboxyl nucleophilic reaction proceeds at a rate that has no precedence in analogous chemical reactions. This same general point can also be made in regard to hydrolysis of CM with which attack of Glu-270 may be rate determining. Thus, the enzymatic nucleophilic reaction very likely involves both Glu-270 and metal ion participation, although the facility of such a reaction cannot as yet be explained.

Registry No. CPA, 11075-17-5; CPL, 26467-18-5; CM, 54845-98-6; Ni, 7440-02-0; Co, 7440-48-4; Cu, 7440-50-8; Hg, 7439-97-6; Glu, 56-86-0.

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Molecular and Catalytic Properties of Glutathione Transferase μ from Human Liver: An Enzyme Efficiently Conjugating Epoxides†

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ABSTRACT: Glutathione transferases with basic isoelectric points are present in the cytosol of all human livers investigated. Adults as well as fetuses contain such basic proteins, referred to as transferases α–ϵ. Some adult (about 60%), but no fetal, livers have a different enzyme, glutathione transferase μ, with an isoelectric point at pH 6.6 [Warholm, M., Guthenberg, C., Mannervik, B., & von Bahr, C. (1981) *Biochem. Biophys. Res. Commun. 98*, 512–519]. This enzyme has also been found in adult adrenal glands. Chemical and physical properties distinguish three major types of human transferases: transferase μ, transferases α–ϵ, and transferase π from human placenta. Transferase μ has two subunits of M_r 26 300; M_r 53 000 and Stokes radius = 3.0 nm were determined independently for the native dimeric protein. Analyses of the amino acid compositions show that the three types of transferases are not interconvertible by posttranslational modifications. Antibodies against any of the human transferases did not cross-react with the other proteins or with rat liver glutathione transferases. Circular dichroism spectra in the near-ultraviolet region are clearly different for the three types of transferases. Estimation of the secondary protein structure from the circular dichroism in the far-ultraviolet region gave 23% α-helix and 25% β-structure for transferase μ. Noteworthy kinetic properties of transferase μ are high specific activities with *trans*-4-phenyl-3-buten-2-one, benzo[*a*]pyrene 4,5-oxide, and styrene 7,8-oxide; pH optimum at 7.5. A random order sequential reaction scheme could explain the steady-state kinetics; experimental data were evaluated by means of nonlinear regression analysis. Transferase μ is highly efficient with benzo[*a*]pyrene 4,5-oxide as substrate: K_m = 0.9 μM, and $k_{cat}/K_m = 3.2 \times 10^7$ min^{-1} M^{-1}. Glutathione derivatives with hydrophobic S-substituents were strong reversible inhibitors: K_i = 0.75 μM (competitive with glutathione) for *S*-*n*-hexylglutathione. Deoxycholate (K_i = 21 μM) and cholate (K_i = 40 μM) were both competitive with the second substrate, 1-chloro-2,4-dinitrobenzene, whereas bromosulfophthalein (K_i = 0.8 μM) and bilirubin (nonlinear inhibition) were noncompetitive with both substrates. Equilibrium binding measured by the quenching of the intrinsic protein fluorescence indicated hyperbolic saturation with bromosulfophthalein (K_d = 1 μM) and bilirubin ($K_d \sim 10$ μM). Transferase μ was irreversibly inactivated by Hg^{2+}, *N*-ethylmaleimide, *N*-phenylmaleimide, 2,4,6-trinitrobenzenesulfonate, and 1-fluoro-2,4-dinitrobenzene. The results indicate strongly that three genetically distinct types of human glutathione transferases exist. The high activity of transferase μ with epoxides may provide better protection against some chemical mutagens and carcinogens to those individuals having transferase μ in their tissues.

The glutathione transferases (EC 2.5.1.18) are a group of multifunctional proteins assumed to be important in the detoxification of many different endogenous and exogenous compounds (Jakoby & Habig, 1980). The most extensive studies of the different forms of the transferases have been performed in rat liver (Jakoby & Habig, 1980), but recently, information about these enzymes in human tissues has also started to appear. Five very similar proteins with basic isoelectric points, referred to as transferases α, β, γ, δ, and ϵ, were first isolated from human liver (Kamisaka et al., 1975). Later, Awasthi et al. (1980) and Koskelo & Valmet (1980) reported the presence of glutathione transferases with acidic isoelectric points in human liver. Similar or identical acidic glutathione transferases have been purified from erythrocytes (Marcus et al., 1978), placenta (Guthenberg et al., 1979; Guthenberg & Mannervik, 1981), and lung (Koskelo et al., 1981; Mannervik et al., 1983). We have shown that an additional form of glutathione transferase exists in the liver of some individuals (Warholm et al., 1980). This form, transferase μ, with a near-neutral isoelectric point, has been purified to apparent homogeneity (Warholm et al., 1981a). We now

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