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Relaxation Spectra of Proteinases. Isomerizations of Carboxypeptidase A (Cox) and (Anson)†

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ABSTRACT: Carboxypeptidase A (Cox) isomerizes at 25° and at neutral pH values in the absence of substrates. The relaxation effect of largest amplitude (τ_1), detected with proton indicators by the equilibrium temperature-jump method, is characterized by pH-dependent rate constants varying from 12 $msec^{-1}$ in acid to 6 $msec^{-1}$ in alkaline solution, and by a single pK_a value near 6. The τ_1 effect is also present in the commercial Worthington enzyme and in the isomers A_γ^{Leu}, A_γ^{Val}, and A_β^{Leu} The pH-dependent behavior of the isomerization can be interpreted in terms of the three-protonation-state model for the enzyme (Auld, D. S., and Vallee, B. L. (1970), *Biochemistry 9,* 4352) and therefore may be linked to the ionization of a group important to the catalytic step of peptide hydrolysis. The transient-state behavior of noncompetitive inhibitors of peptide hydrolysis is correlated with the analogous steady-state behavior. The τ_1 effect is not observed at inhibitor concentrations sufficient to saturate the enzyme under steady-state conditions. Rate constants greater than 12 $msec^{-1}$ are observed in the presence of saturating concentrations of glycyl-L-tyrosine, a pseudosubstrate, and L-phenylalanine, a product and competitive inhibitor of peptide hydrolysis, whereas no change in the relaxation time or amplitude occurs upon addition of L-phenyllactate and L-mandelate, products and competitive inhibitors of ester hydrolysis. The relevance of these results to a proposal of nonidentical binding sites for peptide and ester substrates of carboxypeptidase (Vallee *et al.* (1968), *Biochemistry 7,* 3547) is discussed.

Extensive steady-state kinetic studies of the carboxypeptidase A (Cox)[1] catalyzed hydrolysis of tripeptides over the pH range 4.5–10 and temperature range 5–35° have revealed three protonated forms of the enzyme important to the hydrolysis of peptides ($EH_2 \rightleftarrows EH \rightleftarrows E$). Deprotonation of EH_2 is required for catalysis whereas deprotonation of EH prevents peptide binding (Auld and Vallee, 1970b, 1971). In addition, the use of N-dansylated peptide substrates has allowed studies of enzyme–substrate complexes by stopped-flow fluorescence (Latt *et al.,* 1970, 1972). By this means the mode of action of inhibitors has been assigned directly. Mixed inhibition by a number of agents has been resolved into noncompetitive and competitive components and their pH dependence was determined (Auld *et al.,* 1972).

Any isomerizations of the holoenzyme, as well as studies of transient-state kinetics of the action of an enzyme on substrates, pseudosubstrates, and inhibitors, are essential for a description of a detailed mechanism of an enzyme, as was first fully demonstrated for ribonuclease (Hammes, 1968). In the work reported here, a sensitive temperature-jump apparatus (French and Elwood, in preparation) has been used to detect relaxation effects in carboxypeptidase A (Cox) and (Anson) and the isomers A_γ^{Leu}, A_γ^{Val}, and A_β^{Leu} obtained from Anson enzyme. The behavior of the two main isomerizations, τ_1 and τ_2, that occur near neutrality in the Anson enzyme has been explored as a function of pH and after addition of inhibitors, and a pseudosubstrate, glycyl-L-tyrosine. The pH dependence of the τ_1 isomerization of carboxypeptidase A (Cox) was also determined.

Material and Methods

Apparatus. The detector, a combination photodiode-operational amplifier, of the temperature-jump apparatus (French and Elwood, paper in preparation) was adjusted to give a frequency response (0–20 kHz) and signal-to-noise ratio (typically, 6000) that were adequate for study of the relaxation behavior reported here. The precision of the rate constants fell off above 15 $msec^{-1}$ because of the 20-kHz setting of the amplifier. During alignment of the apparatus care was taken to reduce the amplitude of the only artifact that was observed with unbuffered indicator–salt solutions (rate constant *ca.* 100 $msec^{-1}$) to the broad band noise level of the apparatus (*ca.* 5 mV or 0.00003 *A* unit, 0–30 kHz). Relaxation effects that were used for quantitative calculations were obtained with 4.0° temperature jumps; they varied in amplitude from about 50 to 500 mV, corresponding to transmittance changes less than 0.006*T*.

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[1] The nomenclature used in the text for the various preparations and isomers of carboxypeptidases is that of Petra and Neurath (1969). Mes is an abbreviation for 2-(*N*-morpholino)ethanesulfonic acid.

Contamination. In addition to standard precautions for removal of metal contaminants (Coleman and Vallee, 1960) from aqueous solutions of reagents (extraction with metal-free dithizone solution), from solvents (distillation in metal-free apparatus), from dialysis tubing (leaching with warm distilled water), and from glassware (Thiers, 1957), the following precautions were observed in the present work.

The stainless steel electrodes of the temperature-jump cell were electroplated with nickel and then with gold. The plated items were washed once with dithizone and EDTA solutions and then with metal-free water several times. Dithizone-treated rubber gaskets prevented leakage around the electrodes without the use of any further sealant. Solutions were injected with metal-free plastic disposable tuberculin syringes and the effluent was collected in acid-cleaned polyethylene vials and could be used for another run under a new set of conditions. The cell was flushed with metal-free 1 M NaCl between runs. Contamination by metal ions from the base used for pH adjustments (Baker reagent NaOH pellets) was estimated to be less than 2 nM in all experiments.

Indicators. The indicators were judged pure by thin-layer chromatography done on silica gel (Eastman Chromogram sheet 6060 with fluorescent indicator) in butanol–glacial acetic acid–H_2O (8:1:1). All indicator solutions were extracted with dithizone to remove heavy metals. Solutions of Phenol Red, Thymol Blue, *m*-Cresol Purple, and Phenolphthalein did not inhibit peptidase activity[2] even at concentrations of 0.1 mM. An inhibitory contaminant in Chlorophenol Red was removed by reprecipitation (Orndorff and Sherwood, 1923). The pK_a values for Phenol Red and Chlorophenol Red at 25° and $\mu = 1$ were determined by spectrophotometric titration in buffered solutions.

Enzyme Stock Solutions. Crystals of beef pancreas carboxypeptidase A (Anson) Worthington lot no. COA-9HB, 0DA, 0LX, 1GX, or 2CA were washed with water three times by centrifugation and decantation of the supernatant solution. The crystallized isomers, carboxypeptidase A_β^{Leu}, A_γ^{Leu}, and A_γ^{Val}, were obtained from Anson enzyme by the method of Petra and Neurath (1969). Three-times-recrystallized carboxypeptidase A (Cox) was prepared from beef pancreas acetone powder by the method of Cox *et al.* (1964). Stock solutions of enzymes were stored at 4° and pH 7.5. Under these conditions there was no change in relaxation spectra or in peptidase activity over the period of experimentation. All samples displayed a normal activity of 8000 ± 500 min^{-1} in the CbzGly-L-Phe assay (Auld and Vallee, 1970a).

Other Reagents. Recrystallized indole-3-acetate, phenylacetate, and glycyl-L-tyrosine (Mann assayed); L-phenylalanine, L-mandelate, Tris (Trizma base), and Mes (Sigma); and β-phenyllactate (Ash-Stevens) were used without further purification. Aristar metal-free HCl was obtained from British Drug House Companies Ltd.

Temperature-Jump Measurements. The stock enzyme solution (1 mM) in 3 M NaCl was diluted daily with degassed 0.77 M NaCl to 0.1 mM. In a typical run 1 ml of the diluted enzyme, 1 ml of degassed 1 M NaCl or modifier in 1 M NaCl, and 0.5 ml of degassed 0.1 mM indicator in 1 M NaCl were mixed and adjusted to the desired pH at 25.0° with 0.1 M HCl or 0.1 M NaOH. A radiometer PHM 26 meter was used for pH measurements. Illumination was provided by a tungsten lamp equipped with interference filters (546 nm for Phenol Red and Phenolphthalein indicators and 577 nm for Chlorophenol Red, Thymol Blue, and *m*-Cresol Purple indicators). The microcell, similar to one described by Erman and Hammes (1966), was flushed with 0.2–0.4 ml of sample before each temperature jump was applied. Care was taken to remove products of electrolysis that accumulated near the electrodes and to dislodge bubbles. Effluent enzyme samples retained full activity.

Enzyme solutions were routinely scanned over the time range of 100 μsec to 250 msec, with occasional measurements extending beyond these limits.

Treatment of Data. All rate constants were computed by linear least-squares regression, except for rate constants for the faster of the pair of effects near neutrality in Anson enzyme. The latter were computed by nonlinear least-squares regression using a model of the form

$$A = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$$

in which amplitudes A_1 and A_2, and the faster rate constant, τ_1^{-1}, are the parameters, and τ_2^{-1} is a constant obtained from a prior linear regression of the slower effect.

The theoretical curves of Figures 2–4 were generated from best values of parameters in the rate equations, using nonlinear least-squares regression analysis applied to the mean values of the rate constants at constant pH and using weights equal to the square root of the number of observations at each pH. The errors in best values of τ^{-1} calculated from the best values of the parameters for the two main relaxation effects in this study are about 5% (nonlinear confidence limits, approximate 70% confidence level). When observed values of τ^{-1} are reported for modifiers or other reagents in the present study, the standard error of estimate at 70% confidence varies from 10 to 30% of the mean, which is usually obtained with three to five observations.

Results

Relaxation Spectra of Carboxypeptidase A (Anson) and Carboxypeptidase A (Cox). Temperature-jump measurements with Chlorophenol Red and Phenol Red as indicators were performed over the pH range 4.9–7.7 at a final temperature of 25.0°, at an ionic strength of 1.0 (NaCl) and at enzyme concentrations of 10–100 μM. The pH–rate constant profiles are obtained for enzyme concentrations of 34–51 μM and 20 μM indicator. Two relaxation effects are predominant in the pH

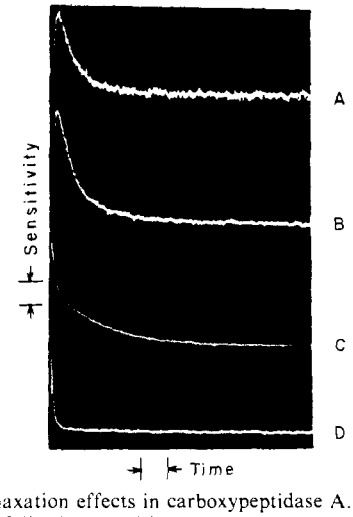

FIGURE 1: Relaxation effects in carboxypeptidase A. The traces were obtained at the following conditions: enzyme concentrations 45–51 μM; indicator concentrations 20 μM, Chlorophenol Red below pH 6.2, Phenol Red above pH 6.2; signal for 6% transmittance change, 2–5 V; temperature pulse 4° to give a final temperature of 25°. The pH, sensitivity in mV/cm, and time axi in msec/cm are noted in parentheses for each trace: A, τ_1 (Anson) (5.8, 20, 0.2). B, τ_1 (Cox) (6.5, 50, 0.2); C, τ_1 and τ_2 (Anson) (6.9, 50, 2); D, τ_1 (Cox) (6.7, 100, 2).

[2] The concentration of the tripeptide substrate, CbzGlyGly-L-Leu, was adjusted to $K_m/20$ at pH 7.5 (Auld and Vallee, 1970b) to test for possible inhibitory effects of K_m and k_{cat} simultaneously.

TABLE I: Independence of τ_1^{-1} and τ_2^{-1} on Enzyme and Indicator Concentrations at pH 6.75.

$[E_T]$, μM	$[In_T]$, μM	τ_2^{-1}, msec^{-1}	τ_1^{-1}, msec^{-1}
10	6	0.21	4.8
36	20	0.21	4.6
43	20	0.25	3.6
50	20	0.21	4.5
51	20	0.24	3.8
90	60	0.25	4.7
100	60	0.21	4.6

range 5-7 and the time range 100 μsec to 250 msec with carboxypeptidase A (Anson), but only one with carboxypeptidase A (Cox) and the γ and β isomers obtained from the Anson enzyme.

The faster effect (Figure 1A,B), τ_1, present in all enzymes, displays an exponential decay in the direction of increasing absorbance (increasing pH) subsequent to rapidly equilibrating steps resulting in a net dissociation of protons from the enzyme-indicator system (protolytic shift). As the pH is increased from 4.9 to 7.3, τ_1^{-1} decreases from about 10 to 3 msec^{-1} for carboxypeptidase A (Anson) and from 11 to 6 msec^{-1} for carboxypeptidase A (Cox).

The slower effect, τ_2 (Figure 1C), is observed in all five lots of carboxypeptidase A (Anson) studied. The direction of the decay is the same as for τ_1. Rate constants increase from 180 to 400 sec^{-1} as pH is increased from 6 to 7.7. The maximum amplitude, 150 mV, occurs near pH 6.9 when Phenol Red is the indicator. At pH 7.7 the amplitude of the effect is only 30 mV and is too small to measure at pH 8, either with Phenol Red, Thymol Blue, or *m*-Cresol Purple. The amplitude and time constant of the relaxation are identical within experimental error at pH 6.8 for all lots of Anson enzyme examined. Neither recrystallization nor extensive dialysis against Tris-buffered 1 M NaCl (pH 7.5) (final concentration of Tris, 10^{-4} M) eliminates the τ_2 effect. The τ_2 effect appears to be unimportant to the enzyme mechanism since it is absent from the fully active isomers obtained from Anson enzyme. Characterization of τ_2 in the following sections is required for an accurate characterization of τ_1 in Anson enzyme only. At the extremes of the pH range three additional relaxation effects were discovered but were examined in less detail.[3]

Concentration Independence of the τ_1 and τ_2 Relaxation Effects. The possible dependence of τ_1^{-1} and τ_2^{-1} on enzyme concentration and enzyme plus indicator concentrations were explored at a pH where both effects were prominent. No dependence on either concentration was found when both concentrations are varied 10-fold (Table I).

In addition to the above results, which exclude concentration-dependent mechanisms as being responsible for the observed relaxations, two corollary experiments also support the exclusion of indicator binding. The amplitudes of both effects in carboxypeptidase A (Anson) decrease 60% in 0.01 M Tris buffer (pK_a = 8.4, μ = 1) at pH 6.5 and are eliminated in 0.01 M Mes buffer (pK_a = 6.3, μ = 1) at pH 6.7. Moreover, there is no detectable difference in the concentration of Phenol Red (50 μM) inside and outside of a dialysis sac containing 45 μM enzyme at pH 6.8 after equilibrium is reached. This behavior is inconsistent with indicator binding but is consistent with the observed effects being due to isomerization mechanisms.

[3] Only lot CoA-9HB was used to explore the behavior of these relaxations in Anson enzyme. A relaxation effect, which displays an exponential decay in the direction of decreasing absorbance, is observed at pH values near 5. Its amplitude is *ca.* 100 mV and its rate constant is *ca.* 0.1 msec^{-1}. A relaxation in the same absorbance direction as τ_1 is detected near pH 9 and is characterized by a low amplitude and rate constant of 0.5 msec^{-1}. Finally, a relaxation with an absorbance direction opposite to τ_1 is observed over the pH range 7.7-8.6. Its amplitude is *ca.* 20 mV and its rate constant *ca.* 30 sec^{-1}.

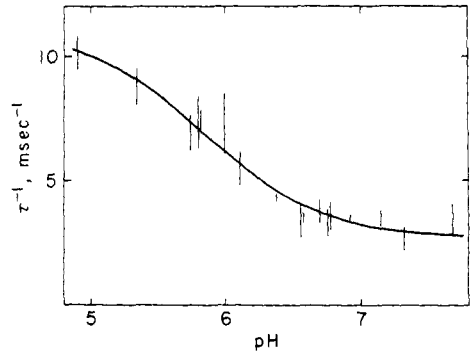

FIGURE 2: Dependence of τ_1^{-1} for carboxypeptidase A (Cox) on pH. The solid line is generated from eq 1 and the values of the parameters obtained by weighted nonlinear least squares analysis of 14 mean values of τ_1^{-1} derived from 67 individual observations of τ_1^{-1} at enzyme concentrations of 45 and at 20 μM Phenol Red (above pH 6) or Chlorophenol Red (below pH 6). The error bars represent standard errors of estimate at the 70% confidence level, corrected for small numbers (2-10) of observations with the *t* statistic. The best values of the parameters and their nonlinear confidence limits at the (approximate) 70% confidence level are: k_1, 5760 ± 260 sec^{-1}; k_{-1}, 6610 ± 730 sec^{-1}; pK_1, 5.68 ± 0.11.

pH-Rate Constant Profiles. The pH-rate constant profile of τ_1^{-1} for Cox enzyme fits a single pK_{app} value according to an equation of the form

$$\tau_1^{-1} = k_1 + k_{-1}(1 + K_1/a_H)^{-1} \qquad (1)$$

as shown in Figure 2. In this equation, k_1 and k_{-1} are rate constants, K_1 is an acid dissociation constant, and a_H is hydrogen ion activity. The same equation applies to the Anson enzyme but a slightly different value of pK_1 is obtained (Figure 3). The minimal mechanism that corresponds to eq 1 is

$$EH' \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EH \overset{K_1/a_H}{\rightleftharpoons} E$$

where the isomerization step equilibrates slowly and the acid-dissociation step equilibrates rapidly. In this mechanism τ_1^{-1} decreases with *increasing* pH to a limiting value, k_1, as observed. Since the τ_1 process everywhere equilibrates rapidly

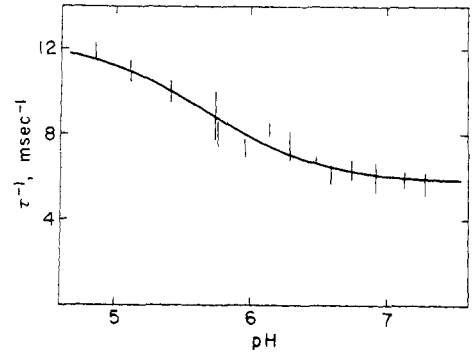

FIGURE 3: Plot of τ_1^{-1} for the Anson enzyme against pH. The average enzyme concentration was 47 μM and indicator concentration was 20 μM. Chlorophenol Red was used for pH values <6.56 and Phenol Red for pH values >6.56. The values of τ_1^{-1} at pH 6.56 were measured with each indicator. Best values of parameters in regression of 17 mean values derived from 73 observations are k_1, 2710 ± 260 sec^{-1}; k_{-1}, 8340 ± 710 sec^{-1}; pK_1, 5.85 ± 0.08.

TABLE II: Effects of Modifiers on Carboxypeptidase A (Anson) at Neutrality.

Modifier	Concn (mM)	τ_1^{-1} (msec^{-1})	τ_1 Amplitude % of Control	τ_2^{-1} (msec^{-1})	τ_2 Amplitude % of Control
Indole-3-acetate	0.39		0	0.25 ± 0.02	100
Phenylacetate	2.0	*a*	10	0.22 ± 0.02	60
L-Mandelate	4.0	3.9 ± 0.2	100	0.27 ± 0.03	100
L-Phenyllactate	2.0	3.8	100	0.27 ± 0.01	100
L-Phenylalanine	20.0	*b*	0	0.22	30

[a] The rate constant for a new effect, in the direction of decreasing absorbance, was 1.6 msec^{-1}. [b] The rate constant for a new effect, in the direction of increasing absorbance, was 14 msec^{-1}.

with respect to the τ_2 process, the pH dependence of τ_1^{-1} is unaltered by the pH dependence of τ_2^{-1}.

The pH–rate constant profile of τ_2^{-1} for Anson enzyme also fits a single pK_{app} value (Figure 4), but the form of the equation is

$$\tau_2^{-1} = k_2 + k_{-2}(1 + a_H/K_2)^{-1} \quad (2)$$

which corresponds to a slowly equilibrating isomerization step followed by a rapidly equilibrating protonation step. Here τ_2^{-1} decreases with *decreasing* pH to a limiting value, k_2, as observed. It is not possible to exclude chemical coupling[4] (*i.e.*, τ_1 species preequilibrating with τ_2 species) by analysis of pH–rate constant profiles because the large difference in pK_{app} values, 1.4 units, in the τ_1^{-1} and τ_2^{-1} pH profiles leads to a small difference in the shape of the theoretical curves obtained from models for chemical coupling and uncoupling. However, because of this large difference in pK_{app} values, the rate constants and dissociation constant do not differ significantly from the values derived using eq 2.

Relaxation Kinetics of Carboxypeptidase A_γ^{Val}, A_γ^{Leu}, *and* A_β^{Leu}. The relaxation effects of the major constituents of Anson enzyme A_γ^{Val}, A_γ^{Leu}, and A_β^{Leu} were examined. The amplitude of the τ_1 process is essentially identical with that of the native Anson enzyme. As expected, the values of τ_1^{-1} for the isomers correspond to those of the Anson enzyme rather than the Cox enzyme. For A_γ^{Leu}, at pH values of 6.66 and 6.97, the observed values are 4.0 and 3.0 msec^{-1} (calcd for Anson enzyme, 3.8 and 3.3 msec^{-1}; calcd for Cox enzyme, 6.4 and 6.1 msec^{-1}). For A_γ^{Leu}, at pH values of 6.72 and 6.92, the observed values are 4.1 and 4.1 msec^{-1} (calcd for Anson enzyme 3.7 and 3.4 msec^{-1}; calcd for Cox enzyme, 6.3 and 6.1 msec^{-1}).

The τ_2 process is essentially absent (≤5 mV) from the isomers obtained from the Anson enzyme. In addition, this effect is not restored in an equimolar mixture of the A_γ^{Leu} and A_γ^{Val} isomers.

Effect of Inhibitors. Values of K_I were estimated at pH 6.8 by steady-state methods (D. S. Auld, in preparation). This pH was chosen as optimal for simultaneous study of both relaxations in the Anson enzyme. Inhibitor concentrations about 10-fold above K_I were chosen for the analysis presented in Table II. Under these conditions, both phenylacetate and indole-3-acetate, noncompetitive inhibitors of peptide hydrolysis, abolish the τ_1 effect without significantly changing the amplitude or rate constant for the τ_2 process (calcd from eq 2 to be 0.25 msec^{-1}) (Table II). As expected, at lower ratios of $[I]/K_I$ there is a partial reduction in the amplitude of τ_1. For example, at an $[I]/K_I$ value of 1.1 with indole-3-acetate, the τ_1 amplitude is reduced by 70%. L-Phenyllactate and L-mandelate, which are products and competitive inhibitors of ester hydrolysis, do not affect the amplitude or rate constant for τ_1 (calcd from eq 1 to be 3.6 msec^{-1}) or τ_2. The rate constant characteristic of τ_1 is not observed in the presence of L-phenylalanine, a product and a competitive inhibitor of peptide hydrolysis, while the rate constant of the τ_2 process remains constant. However, a relaxation effect in the same absorbance direction as τ_1 and having a rate constant of 14 msec^{-1} is observed.

Pseudosubstrate. The relaxation spectrum of carboxypeptidase A (Anson) saturated with 4 mM glycyl-L-tyrosine was measured at 25° over a period of 30 min. Conditions were chosen such that less than one-tenth of the substrate is hydrolyzed during the relaxation experiment and, by steady-state criteria, the products are at concentrations low enough not to interfere with the reaction. The rate constant characteristic of τ_1 is not observed in the presence of glycyl-L-tyrosine; instead, an effect in the same absorbance direction as τ_1 with a rate constant of 17 msec^{-1} is observed. The value of τ_2^{-1} remains constant.

Discussion

The τ_1 relaxation effect is present in all enzymatically active enzyme preparations examined thus far. This effect is readily apparent in the pH range 5–7 and is a relaxation of the monomeric enzyme as shown by the enzyme concentration independence of τ_1^{-1} (Table I). The lack of dependence of τ_1^{-1} on the concentration of enzyme plus indicator excludes mechanisms involving direct interaction of enzyme and indicator (Table I). Indicator binding is also ruled out by the equilibrium dialysis experiment with Phenol Red. In addition, a direct but slow (*ca.* 10^7 M^{-1} sec^{-1}) proton transfer between enzyme and indicator would require a mechanism where the pK_a of the indicator ap-

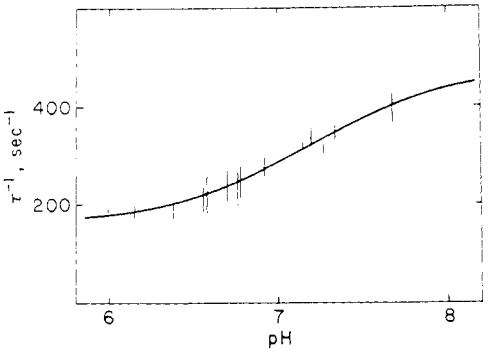

FIGURE 4: pH dependence of τ_2^{-1} for carboxypeptidase A (Anson). The theoretical curve was generated by substitution in eq 2 of the best values of parameters k_2, 159 ± 7 sec^{-1}; k_{-2}, 322 ± 18 sec^{-1}; pK_2, 7.20 ± 0.05, which were obtained by analysis of 14 mean values of τ_2^{-1} derived from 72 individual observations of τ_2^{-1}.

[4] The chemical evidence, *i.e.*, effects of inhibitors on the τ_1 and τ_2 relaxation and absence of τ_2 in carboxypeptidase isomers, strongly suggest that the relaxation effects occur at independent sites which also may be chemically uncoupled.

pears in the pH dependence of τ_1^{-1}. This behavior is not observed since each pH–τ_1^{-1} profile is a curve that fits a single pK (Figures 2 and 3) although these profiles have been obtained using indicators having different pK_a values, namely, Phenol Red (pK_a = 7.53, μ = 1) and Chlorophenol Red (pK_a = 5.89, μ = 1). The τ_1 relaxation effect is therefore best described as an isomerization of the monomeric enzyme.

The τ_1 isomerization is very sensitive to inhibitors of enzyme action and a pseudosubstrate. The rate constant characteristic of τ_1 is not observed in the presence of a number of inhibitors such as L-phenylalanine, phenylacetate and indole-3-acetate as well as the pseudosubstrate, glycyl-L-tyrosine (Table II). Moreover, in the presence of L-phenylalanine and glycyl-L-tyrosine, relaxation effects with rate constants greater than $k_1 + k_{-1}$ are observed. The pH dependence of τ_1^{-1} suggests that the τ_1 isomerization may be linked to the ionization of a group important to the catalytic step of peptide hydrolysis.

The rate constants for the forward and reverse direction of the τ_1 isomerization are both an order of magnitude higher than the maximal rate constant for a good substrate of the enzyme (*e.g.*, Cbz(Gly)$_2$-L-Phe, 120 sec^{-1}). Hence, it is possible for the τ_1 isomerization to be kinetically coupled to steps on the catalytic pathway. If the acid leg of the reaction scheme proposed by Auld and Vallee (1970b) is modified only by an isomerization between EH_2 species, EH_{2a} and EH_{2b}

$$EH_{2a} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EH_{2b} \overset{K_3/a_H}{\rightleftharpoons} EH$$

the apparent pK of free enzyme, pK_{EH_2}, that would obtain in steady-state analysis of the pH dependence of k_{cat}/K_m can be written directly as

$$pK_{EH_2} = pK_1 + \log [1 + (k_{-1}/k_1)] \quad (3)$$

where K_1, k_1, and k_{-1} have been defined by eq 1.

The nonlinear confidence limits (70%) on the pK_{EH_2} computed from the model of eq 3 can be obtained by a propagation of error treatment. The computed values of pK_{EH_2}, 6.01 ± 0.11 for the Cox enzyme and 6.46 ± 0.09 for the Anson enzyme, are in good agreement with the results of steady-state analysis. Auld and Vallee (1970b) found values for pK_{EH_2} to be 6.18 ± 0.07 and 6.14 ± 0.05 for the Cox enzyme acting on the tripeptide substrates CbzGlyGly-L-Leu and CbzGlyGly-L-Val. Values of 6.5 and 6.9 have been reported for the Anson enzyme acting on the substrates *O-trans*-cinnamoyl-L-β-phenyllactate (Hall *et al.*, 1969) and *O*-acetyl-L-mandelate (Carson and Kaiser, 1966), respectively. Hence, it is possible to incorporate the transient-state and steady-state pH-dependent behavior into a single mechanistic scheme. Steady-state studies of the pH dependence of inhibitors such as phenylacetate show them to interact much more strongly with the EH_2 than the EH form of the enzyme, resulting in a shift in pK_{EH_2} when the enzyme is saturated with inhibitor (Auld *et al.*, 1972). Further transient-state studies will be needed to determine whether the shift in pK_{EH_2} truly represents a shift in the pK_1 in the above mechanism, and whether a slow, uncoupling isomerization is introduced between the τ_1 effect and EI or EIS complexes.

Evidence in Support of Different Binding Sites for C-Terminal Products of Peptide and Ester Substrates. The fact that products of peptide and ester hydrolysis, L-phenylalanine and L-phenyllactate, have differing effects on τ_1 is also of interest mechanistically. Results of X-ray analysis of zinc carboxypeptidase (Libscomb *et al.*, 1968) and nmr studies of manganese carboxypeptidase interacting with L-phenylalanine and L-phenyllactate (Quiocho *et al.*, 1971) have been interpreted to imply that these two agents bind to the enzyme in identical manners. However, the present studies show that this binding must differ in some way, since the rate constant characteristic of τ_1 is not observed in the presence of phenylalanine; instead an effect with a rate constant (14 msec^{-1}) higher than $k_1 + k_{-1}$ (11 msec^{-1}) is observed. In contrast, both of the α-hydroxy acids, L-phenyllactate and L-mandelate, have no effect on the rate constant or amplitude of the τ_1 process. These results are consistent with the proposal of nonidentical binding sites for peptide and ester substrates of carboxypeptidase (Vallee *et al.*, 1968).

Comparison with the Behavior of Ribonuclease. The principal isomerizations of carboxypeptidase A and ribonuclease (French and Hammes, 1965), which are both kinetically related to the catalytic mechanism, share several common characteristics. For example, under similar conditions of pH, enzyme concentration, and indicator concentration, a temperature pulse causes a rapid drop in pH (protolytic shift), followed by a net *increase* in pH during the relaxation process. In each case the relevant minimal mechanism corresponds to proton dissociation from only one of the two isomers. The nondissociable isomer is assumed to correspond to an inactive form by virtue of correlation of the transient-state pK of the dissociable isomer with the steady-state pK of free enzyme. The transient-state pK's are similar (carboxypeptidase A (Anson), 5.9 at μ = 1.0; ribonuclease A, 6.1 at μ = 0.1). In addition, substrates (glycyl-L-tyrosine and cytidine 2′,3′-cyclic phosphate) and products (L-phenylalanine and cytidine 3′-monophosphate, respectively) cause relaxation effects to appear that are outside of the range of rate constants characteristic of the native isomerization process.

Meadows *et al.* (1969) have interpreted results of nmr, X-ray, and temperature-jump studies of ribonuclease, and have assigned the dissociable group involved in the τ_1 isomerization (pK = 6.1, solvent H_2O, 25°, 0.1 M KNO_3) to histidine 48 (pK = 6.4, solvent D_2O, 32°, 0.2 M NaCl) chiefly on the basis of the influence of pH and inhibitors on the position and line width of the C(2)–H peak. The easy resolution of this peak below pH 6 is partly ascribed to a fortuitous interaction of tyrosine-25 with histidine-48. In contrast, there is not yet any direct evidence that a histidine residue is involved in the τ_1 isomerization of carboxypeptidase A, nor is it clear whether the much more complex histidine spectra of carboxypeptidase can be resolved. However, the ready availability of a number of specifically labeled active-center derivatives of carboxypeptidase may aid in the identification of loci at which this isomerization occurs.

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Cotransport of Organic Solutes and Sodium Ions in the Small Intestine: a General Model. Amino Acid Transport†

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ABSTRACT: A general, allosteric, noncompulsory model has been developed that may in principle be used to explain in a unified manner all organic solute and Na^+ cotransport systems in the small intestine, as well as some other carrier or enzyme systems, *e.g.,* brush border sucrase. This general model, simplified to ignore V (capacity) effects, is used to explain and fit new experimental results on cycloleucine (1-aminocyclopentanecarboxylic acid) transport in guinea pig jejunum and ileum, as well as published results (Curran *et al.,* 1967, *J. Gen. Physiol. 50,* 1261) on L-alanine transport in rabbit ileum. Equations have been developed to calculate the pertinent dissociation constants for both amino acids and Na^+. Quantitative comparisons between the results obtained indicate an essentially identical, noncompulsory mechanism for amino acid and Na^+ cotransport in the small intestine of guinea pig and rabbit.

Sodium ion is a specific activator of the transport of a variety of substances across several cell membranes (for review, see Schultz and Curran, 1970). A striking example is that of the small intestine, where at least ten different classes of solute transport mechanisms have been shown to require Na^+. The list includes the transport mechanism for sugars, amino acids (which in turn may be subdivided into four separate classes), bile salts, uracil, sulfate, phosphate, calcium, ascorbic acid, biotin (Berger *et al.,* 1972), and riboflavine (Rivier, 1973). There is also much evidence that the energy inherent in the existence of a Na^+ gradient across the brush border membrane can be used to produce an asymmetric (uphill) transport of organic solutes by virtue of the coupling between the two transports (for Na^+ and solute, respectively) at the level of a common carrier. The term, cotransport, specifically refers to this coupling.

Pros and cons of the so-called sodium gradient hypothesis have been amply discussed (Schultz and Curran, 1970; Heinz, 1972; Kimmich, 1973). In this series of papers, however, attention will be focused, not on the question of the source of energy for organic solute uphill transport, but rather on the mechanism of cotransport that implies a reciprocal activating relationship between Na^+ and organic solutes. As our theoretical basis we will use a general, allosteric,[1] noncompulsory model in which the carrier is defined as bifunctional, *i.e.,* as having two separate but functionally related binding sites, one for organic solute and one for Na^+. As first proposed by Alvarado (1966, 1967), the relationship between the two sites will be considered similar to that of an allosteric enzyme with one substrate and one allosteric (modifier) site. In fact, the model is suitable for application to some enzymes, *e.g.,* the case of Na^+ activation of brush-border sucrase (Mahmood and Alvarado, to be published). However, in this particular series dealing with transport carriers, and because of the nature of the cotransport concept, substrate and modifier are interchangeable, the model being entirely symmetrical. After defining the general model, we will show how it fits our experimental results on cycloleucine transport in guinea pig intestine, as well as those found by others using L-alanine and rabbit ileum (Curran *et al.,* 1967). Subsequent papers will be devoted to other cotransport systems, *e.g.,* that for sugars and Na^+.

Our ultimate goal in this project is: (1) to ascertain whether the same general model can accurately describe the cotransport of various organic solutes and Na^+ in the small intestine of mammals (and perhaps other vertebrates); and (2) if the above is found to be the case, to ascertain whether the Na-binding sites involved in amino acid and sugar transport activation are the same; *i.e.,* whether Na^+ acts a *general* rather than as an *individual* activator of solute transport in the intestine (Alvarado, 1972). An affirmative answer to this question would open

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[1] We use the term *allosteric* in its original sense, namely, "*indirect* interactions between *distinct* specific binding sites" (Monod *et al.,* 1965).