Kinetics of Carboxypeptidase A. pH and Temperature Dependence of Tripeptide Hydrolysis*

David S. Auld† and Bert L. Vallee

ABSTRACT: The carboxypeptidase A catalyzed hydrolysis of carbobenzoxyglycylglycyl-L-phenylalanine, -leucine, and -valine and of benzoylglycylglycyl-L-phenylalanine have been studied over the pH range 5–10 and the temperature range 5–35°.

Over the entire temperature range investigated the pH dependent hydrolyses can be described by a kinetic scheme requiring minimally two critical ionizations of the free enzyme, $EH_2 \rightleftharpoons EH \rightleftharpoons E$, and of one in the enzyme–substrate complex, $EH_2S \rightleftharpoons EHS$, with only EHS being catalytically active. The thermodynamic parameters for K_{EH_2}, K_{EH}, and K_{EH_2S} have been determined kinetically and compared to those for side-chain groups in peptides and other enzyme systems. The parameters calculated for K_{EH_2} are intermediate between the values expected for carboxyl and imidazole groups of peptides ($\Delta H_i = 3.1$ kcal mole^{-1}; $\Delta S_i = -18$ eu). The analogous values for K_{EH_2S} closely resemble those of an imidazole group of free histidines ($\Delta H_i = 6.5$ kcal mole^{-1}; $\Delta S_i = -7$ eu). The enthalpy and entropy of the ionization which control binding of the peptide substrates, K_{EH}, are closely similar to those for tyrosyl residues ($\Delta H_i = 5.4$ kcal mole^{-1}; $\Delta S_i = -24$ eu). The ionization of a coordinated water molecule or imino group of imidazole are also considered as possibilities responsible for either one of these ionizations, but on the basis of presently available data final conclusions cannot be drawn in this regard.

While kinetic anomalies have hampered mechanistic studies of the carboxypeptidase A catalyzed hydrolysis of dipeptides and acylamino acids, the hydrolysis of such tripeptide substrates as carbobenzoxy- or benzoylglycylglycyl-L-phenylalanine, -leucine, or -valine (Auld and Vallee, 1970a) is devoid of substrate and product activation. Thus the effect of pH, temperature, and inhibitors on their hydrolysis can be examined in a more mechanistically meaningful manner.

The hydrogen ion dependence of their hydrolysis can be described by a reaction scheme requiring minimally two critical ionizations of the enzyme. The first ionization ($EH_2 \rightarrow EH$; $pK_{EH_2} = 6.2$) results in active enzyme, and the binding of peptide substrates is only slightly dependent on this ionization. The second ionization ($EH \rightarrow E$; $pK_{EH} = 9.0$) markedly reduces peptide binding (Auld and Vallee, 1970b).

Since nearly 3 pH units separate pK_{EH_2} and pK_{EH}, their temperature dependencies can be determined quite accurately. This study compares the kinetically determined enthalpies and entropies of ionization, ΔH_i and ΔS_i, respectively, for the groups whose ionizations characterize the pH–rate profiles with those for side-chain groups in model peptides and other enzyme systems. Assuming equivalence between such data, tentative assignments have been made. A preliminary report on this work has appeared (Auld, 1969).

Materials and Methods

Beef Pancreas Carboxypeptidase. Three times recrystallized zinc carboxypeptidase A was prepared from beef pancreas acetone powder by the method of Cox *et al.* (1964).[1] The crystals were washed three times with deionized distilled water and dissolved in 1 M NaCl–0.05 M Tris (pH 7.5) to yield stock solutions of 2×10^{-4} M. The peptidase and esterase activities expressed as turnover numbers were 8000 ± 500 min^{-1} and 6500 ± 500 equiv H^+/min per mole of enzyme, at pH 7.5, 25° when assayed using the standard substrates of 0.02 M CbzGly-L-Phe and 0.01 M hippuryl-*dl*-β-phenyllactic acid. Although different preparations of enzyme were used, the same preparation was always employed for a particular series of experiments.

The experimental procedures used for preparing metal-free glassware and buffers have been detailed in a previous communication (Davies *et al.*, 1968).

Substrates. Peptides were either synthesized as described in a previous report (Auld and Vallee, 1970a) or purchased from Cyclo Chemical Corp.

The peptides were recrystallized until the free amine present was found to be less than 0.1%. The products of their carboxypeptidase catalyzed hydrolysis were determined by thin layer chromatography on silica gel (Eastman Chromogram Sheet 6060 with fluorescent indicator), using butanol–glacial acetic acid–water (4:1:1, v/v). In all cases only the first amino acid was cleaved in the reaction. The amount of L-amino acid formed was always within 2% of theory over the pH range 5–11 as determined by ninhydrin analysis

Activity Measurements. The concentration of the unblocked amino acid formed in the hydrolysis was determined by either of two automated ninhydrin methods, both of which have been described previously (Auld and Vallee, 1970a,b). The assays are sensitive enough to need no more than 1×10^{-5} M products formed for the direct assay method or 1×10^{-4} M product for the aliquot withdrawal method. Such concentrations of products had no effect on the initial rates under the conditions employed here. Mes,[2] Hepes, Tris, and ammediol

* From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts. *Received March 2, 1971.* This work was supported by Grant-in-Aid GM-15003 from the National Institutes of Health of the Department of Health, Education, and Welfare.

† Fellow of the National Cancer Institute.

[1] Carboxypeptidase A referred to in the text will be carboxypeptidase A (Cox) unless otherwise specified (Petra and Neurath, 1969).

[2] The abbreviations for buffers used are: Ammediol, 2-amino-2-methyl-1,3-propanediol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid.

buffers (0.05 M) were used for holding pH constant. The buffers chosen had no effect on the hydrolysis of the peptides.

A Radiometer pH M 26 scale-expanded pH meter was used for measuring pH's before and after assays. The meter was standardized at the appropriate temperature with Harleco pH 4 and 7 reference buffers. The pH 10 reference buffer read within 0.05 unit of theory using this procedure. In no case was an assay used in which the drift in pH during the reaction exceeded 0.04 pH unit. At alkaline pH's corrections were made for the high sodium content (1 M NaCl) of the assay mixtures. The temperature was controlled to ±0.1°. Over the temperature range 5–35° changes in pH of the Mes and Hepes buffers were 10% lower than the values determined by Good *et al.* (1966).

Determination of Kinetic Parameters. A curve-fitting procedure was used to determine k_2 and K_{EH_2S} from eq 2 and k_2/K_S, K_{EH_2}, and K_{EH} from eq 3. The parameters of the appropriate equation were allowed to take on several values over a predetermined range. The criteria for the best fit were threefold: first, the sum of calculated velocities that differed from the observed values by more than three times the observed error was made a minimum (n_{G3}). Second, the sum that differed by less than twice the error was made a maximum (n_1 plus n_2). Last, the average per cent deviation, D, of calculated values, C, from experimental values, O, was made a minimum (eq 1)

$$D = \frac{100}{N}\sum_{i=1}^{n}\frac{C_i - O_i}{O_i} \qquad (1)$$

where N is the number of data points. The error tests, n_1, n_2, n_3, and n_{G3}, given in the tables denote the number of points within 5, 10, 15, and >15% of the theoretical values calculated from the appropriate equation. An SDS 940 computer aided in the curve-fitting procedure. For linear regression analysis the error in the intercept and slope was calculated from the standard error of estimate, standard deviations, and the Student t distribution at the 70% confidence level.

Results

It has been shown previously that the pH dependence of k_{cat} and k_{cat}/K_m for peptide hydrolysis is given by eq 2 and 3

$$k_{cat} = \frac{k_2}{1 + a_H/K_{EH_2S}} \qquad (2)$$

$$\frac{k_{cat}}{K_m} = \frac{k_2/K_S}{(1 + a_H/K_{EH_2} + K_{EH}/a_H)} \qquad (3)$$

(Auld and Vallee, 1970b), where k_2, K_S, K_{EH_2}, K_{EH}, and K_{EH_2S} are defined in the reaction scheme (Figure 1) and k_{cat} and K_m have the conventional units (eq 4).

$$V_0/[E_T] = k = \frac{k_{cat}[S]}{K_m + [S]} \qquad (4)$$

Temperature Dependence of pK_{EH_2S}. For both BzGlyGly-L-Phe and CbzGlyGly-L-Phe, K_m varies by no more than 20% over the pH range from 5 to 8, when determined at 25 and 5°, while k_{cat} changes by a factor of 5. The temperature dependence of pK_{EH_2S} can therefore be obtained most easily from pH–rate profiles determined at enzyme saturating concentrations of substrate since under these conditions eq 4 reduces to eq 2.

REACTION SCHEME

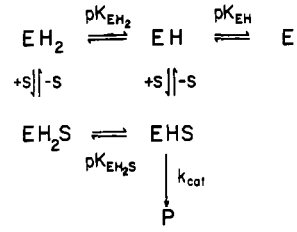

FIGURE 1

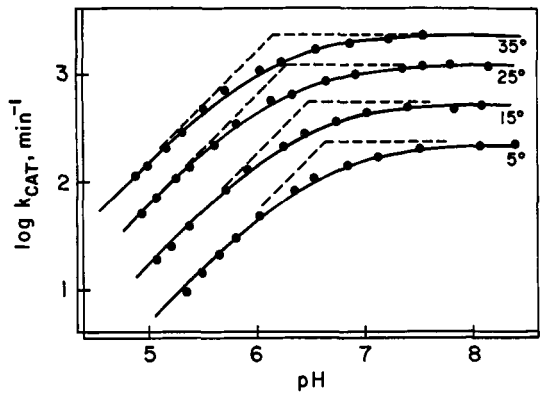

FIGURE 2: pH and temperature dependence of the carboxypeptidase A catalyzed hydrolysis of 0.02 M BzGlyGly-L-Phe. The solid lines are calculated using eq 2 and the best values of K_{EH_2S} and k_2 obtained by the curve-fitting procedure apparent from Table I.

BzGlyGly-L-Phe and CbzGlyGly-L-Phe were employed at concentrations 20- and 40-fold above K_m, respectively, and at 5, 15, 25, and 35°. Plots of log k *vs.* pH for 0.02 M BzGlyGly-L-Phe hydrolysis are shown in Figure 2. For each temperature the intercepts of the dashed lines with slopes of one and zero determine the pK_a' (Dixon, 1953).

For each temperature, the curve-fitting procedure, described in the Experimental Section, results in good fits to the data only over a very narrow range of pK_a' values, usually no greater than ±0.02 pH unit (Table I). However, variation of the temperature from 5 to 35° changes the pK_a' by approximately a half pH unit.

These pK_a' values vary linearly with the reciprocal of absolute temperature. The enthalpy of ionization, ΔH_i, calculated from the slope of this line, is 6.5 kcal/mole and the entropy of ionization is −7.0 eu. Using a different preparation of carboxypeptidase A essentially identical sets of thermodynamic parameters for pK_{EH_2S} are found for the hydrolysis of BzGlyGly-L-Phe (0.02 M) and CbzGlyGly-L-Phe (0.01 M) (Table II).

Temperature Dependence of pK_{EH_2} and pK_{EH}. Nearly 3 pH units separate pK_{EH_2} and pK_{EH} which can be measured from velocities obtained at concentrations of CbzGlyGly-L-Leu and CbzGlyGly-L-Val well below their K_m. The initial rate data are given by eq 5.

$$V_0/[E_T] = k = \frac{k_{cat}[S]}{K_m} \qquad (5)$$

The enthalpies and entropies of ionization for the groups whose ionization are thought to characterize these pH profiles

TABLE I: Temperature Dependence of pK_{EH_2S} for BzGlyGly-L-Phe Hydrolysis.[a]

pK_{EH_2S}	k_2 (min^{-1})	Temp (°C)	n_1	n_2	n_3	n_{G3}	D
6.16	2260	35	8	2	2	0	3.9
6.27	1180	25	12	2	0	0	2.8
6.48	530	15	4	6	2	0	6.2
6.64	221	5	6	5	1	0	5.8

[a] 0.05 M Mes–1.0 M NaCl buffer used from pH 4.8 to 7.3; 0.05 M Tris–1.0 M NaCl buffer used from pH 7.3 to 8.5. Equation 1 was used for the determination of the values of k_2 and K_{EH_2S}. The number of the points in the theoretical calculation within 5, 10, 15, and greater than 15% of the observed values are designated as n_1, n_2, n_3, and n_{G3}, respectively. D is the average per cent deviation of calculated values from observed values.

TABLE II: Thermodynamic Parameters for pK_{EH_2S}, pK_{EH_2}, and pK_{EH}.[a]

Peptide	pK_a'	ΔF_i (kcal $mole^{-1}$)	ΔH_i (kcal $mole^{-1}$)	$-\Delta S_i$ (cal deg^{-1} $mole^{-1}$)
BzGlyGly-L-Phe[b]	pK_{EH_2S}	8.59	6.51 ± 0.50	6.99
		8.27	7.18 ± 0.22	3.64
CbzGlyGly-L-Phe	pK_{EH_2S}	8.25	6.12 ± 0.57	7.14
CbzGlyGly-L-Val	pK_{EH_2}	8.49	3.10 ± 1.2	18.1
CbzGlyGly-L-Leu	pK_{EH_2}	8.49	3.42 ± 0.76	17.0
CbzGlyGly-L-Val	pK_{EH}	12.3	5.85 ± 3.6	21.8
CbzGlyGly-L-Leu	pK_{EH}	12.5	5.00 ± 1.6	25.2

[a] The values of ΔH_i were determined from plots of pK_a' *vs.* $1/T$, $\Delta F_i = RT\ 2.303\ pK_a'$, and $-\Delta S_i = (\Delta F_i - \Delta H_i)/T$, where 25° has been chosen for the calculation of ΔF_i and ΔS_i. The errors given for ΔH_i are at the 70% confidence level. [b] The second row of values given here are for a complete analysis done on another preparation of enzyme.

are then obtained from the temperature dependence of these pK_a' values.

Below pH 6 velocities decrease more rapidly than would be predicted from eq 3, an effect which is independent of the temperature or substrate studied (Figure 3). Below pH 6 a 10- to 100-fold molar excess of Zn^{2+} restores activity to a constant value, suggesting that this additional reduction in activity is due to loss of the catalytically important zinc atoms from the enzyme owing to competition of H^+ (Vallee *et al.*, 1960).

The temperature dependences of pK_{EH_2} and pK_{EH} are obtained for CbzGlyGly-L-Leu, 1.92×10^{-4} M, at 5, 15, 25, and 35° and for CbzGlyGly-L-Val, 5.75×10^{-4} M, at 10, 25, and 35°. Table III shows the pK_a' values determined at the respective temperatures together with the error tests. The fit of eq 3 to the experimental data for hydrolysis of CbzGlyGly-L-Val, 5.75×10^{-4} M, over the pH range from 5 to 10 and at 10 and 25° is shown in logarithmic form in Figure 4.

The resultant pK_a' values vary linearly with the reciprocal of absolute temperatures. The enthalpy and entropy of ionizations are +3.1 kcal/mole and −18 eu for pK_{EH} and +5.4 kcal/mole and −24 eu for pK_{EH}, based on calculations combining the data obtained from the hydrolysis of CbzGlyGly-L-Val and CbzGlyGly-L-Leu.

Activation Parameters of Peptide Hydrolysis. The temperature dependence of the pH–rate profiles can also serve to determine the activation parameters for peptide hydrolysis (Table IV). In all cases, linear Arrhenius plots result. The activation parameters for the hydrolysis of BzGlyGly-L-Phe and CbzGlyGly-L-Phe are determined by employing the pH-independent rate constant, k_2 (Table I). For CbzGlyGly-L-Val and CbzGlyGly-L-Leu the rate constants are determined from the values of k_2/K_s, obtained from the best fits of eq 3 to the pH–rate profiles at low substrate concentrations. Since, in the vicinity of pH 7.5, K_m is invariant both as a function of pH and temperature, the temperature dependence of the limiting rate constants determined under these conditions are also good measures of the activation parameters of k_2 (Table IV).

The enthalpies and entropies of activations for these four peptides differ very little, although a slight compensatory effect may be present. In all cases, the *enthalpy of activation* essentially determines the overall rate of reaction.

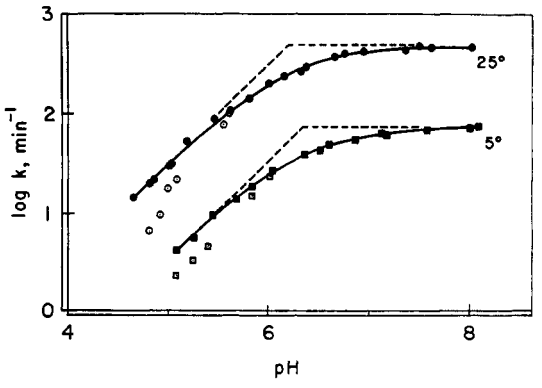

FIGURE 3: The pH dependence of 1.92×10^{-4} M CbzGlyGly-L-Leu at 25° (○,●) and 5° (□,■). Below pH 6.2, the open circles and squares represent hydrolysis in the absence of any added Zn^{2+}, while the solid circles and squares represent hydrolysis in the presence of Zn^{2+} (10^{-5} M). The solid lines are calculated using eq 3 and the best values obtained for pK_{EH_2} and k_2/K_S (Table III).

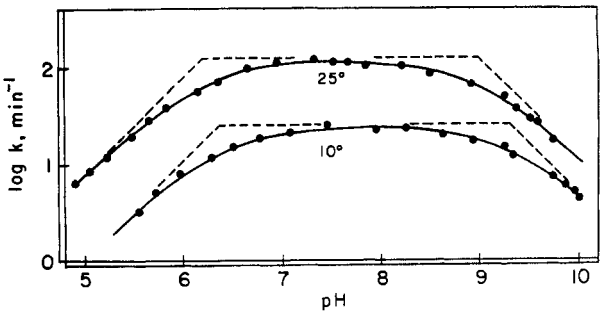

FIGURE 4: pH dependence of CbzGlyGly-L-Val hydrolysis catalyzed by carboxypeptidase A at 10 and 25°. The solid lines are calculated using eq 3 and the best values of pK_{EH_2}, pK_{EH}, and k_2/K_S obtained by the curve-fitting procedure, apparent from Table III.

TABLE III: Temperature Dependencies of pK_{EH_2} and pK_{EH}.[a]

Peptide	Temp (°C)	pK_{EH_2}	pK_{EH}	$(k_2/K_S) \times 10^5$ (min^{-1} M^{-1})	n_1	n_2	n_3	n_{G3}	D
CbzGlyGly-L-Leu	35	6.06	8.92	49.0	9	4	5	4	9.1
CbzGlyGly-L-Leu	25	6.19	9.11	26.0	21	3	5	0	4.3
CbzGlyGly-L-Leu	15	6.25	9.26	11.3	12	4	2	4	7.0
CbzGlyGly-L-Leu	5	6.33	9.30	3.84	15	5	0	1	4.3
CbzGlyGly-L-Val	35	6.17	9.00	5.05	13	8	2	1	6.4
CbaGlyGly-*l*-Val	25	6.19	8.99	2.14	13	7	2	0	5.1
CbzGlyGly-L-Val	10	6.35	9.33	0.45	10	7	1	0	4.6

[a] Assays performed in 1.0 M NaCl–0.05 M Mes, Tris, and Ammediol buffers. Equation 3 was used for the determination of the values of K_{EH_2}, K_{EH}, and k_2/K_S. Statistical evaluation as in Table I.

Discussion

Over the ranges of pH from 5 to 10 and temperature from 5 to 35°, the profiles of steady state pH–rate constants for hydrolysis of the tripeptides here examined can be characterized readily by single ionizations in the acid and alkaline regions. The change in the pK_a' values as a function of different temperatures is relatively large as compared to the error encountered on determining such values at any one given temperature (Tables I and III). The free energies of ionization, ΔF_i, and enthalpies of ionization, ΔH_i, for pK_{EH_2S} are essentially identical for the two substrates BzGlyGly-L-Phe and CbzGlyGly-L-Phe. Similarly, the values of thermodynamic parameters for pK_{EH_2} and pK_{EH} are independent of the substrate when CbzGlyGly-L-Val and CbzGlyGly-L-Leu are employed, as expected if the measurements were to represent ionizations of the same groups in the free enzyme (Peller and Alberty, 1959).

When the pertinent velocity measurements are determined at low substrate concentrations, the thermodynamic parameters might also reflect the stability of the enzyme. At 25° the enzyme is much more stable between pH 6 and 10 than below pH 6 where, in fact, excess metal ion is required to stabilize the enzyme (Auld and Vallee, 1970b). Such effects of both metal ions and substrates on the stability of enzymes are not peculiar to carboxypeptidase, since cofactors and inhibitors are known to stabilize other enzymes against denaturation (Snell and Jenkins, 1959; Fisher and Krebs, 1966) and to stabilize native structure of metalloproteins (Kägi and Ulmer, 1968; Ulmer, 1970; Ulmer and Vallee, 1971). Moreover, chemical modification of an active site residue, *e.g.*, the acylserine-195–chymotrypsin intermediate, is known to stabilize the enzyme toward denaturation (Wooten and Hess, 1960; Bender *et al.*, 1962; Bernhard *et al.*, 1965; Bernhard, 1968). Thus, saturation of the enzyme with substrate might also stabilize the enzyme against denaturation by heat or hydrogen ions.

The kinetically determined heats of ionization may aid in the identification of functional residues involved in catalysis and/or binding, assuming the validity of drawing analogies between enzymes and model systems such as amino acids and peptides. While certain pairs of residues, *e.g.*, carboxyl and imidazolyl or amino and phenoxy groups, have similar free energies of ionization, their enthalpies and entropies of ionization differ significantly (Edsall and Wyman, 1958).

Kinetic studies of tripeptide hydrolysis have shown that ionization of an enzyme species, EH, results in another enzyme form, E, which no longer binds peptide substrates (Auld and Vallee, 1970b). The $pK_a' = 9.0$ derived for the group involved in this transition could be consistent either with the ionization of a metal aquo complex, *i.e.*, $Me(L_n)(H_2O) \rightleftharpoons Me(L_n)(OH) + H^+$, lysyl, or tyrosyl residues. K_{EH} has been shown to be independent of the metal present (Auld and Vallee, 1970b). If the ionization $EH \rightarrow E + H^+$ were to reflect the ionization of a metal–aquo complex such behavior would be distinctly different from that observed for metal complex ions where the ionization constants have been found to be metal dependent (Cotton and Wilkenson, 1966; Bolzan and Arvia, 1962; Perrin, 1962a,b). However, the thermodynamic parameters for the ionization of a coordinated water molecule in multiliganded complexes are not available at present for purposes of comparison with the data obtained here for carboxypeptidase, and, hence, on the basis of current knowledge, final conclusions cannot be drawn concerning the role of a metal–aquo complex in this ionization.

Potentially, lysyl and tyrosyl residues,[3] can be differentiated based on their thermodynamic parameters obtained from

TABLE IV: Activation Parameters for Peptide Hydrolysis by Carboxypeptidase.

Peptide	ΔF^+ (kcal mol^{-1})	ΔH^+ (kcal $mole^{-1}$)	$-T\Delta S^+$ (kcal $mole^{-1}$)
BzGlyGly-L-Phe	15.5	12.7 ± 0.6	2.8
CbzGlyGly-L-Phe	14.6	12.6 ± 0.6	2.0
CbzGlyGly-L-Leu	14.7	13.9 ± 1.8	0.8
CbzGlyGly-L-Val	15.1	15.1 ± 2.6	0.0

[a] The values of E_a were determined from plots of log k_2 or log k_2/K_S *vs.* $1/T$, $\Delta H^+ = E_a - RT$, $-T\Delta S^+ = (\Delta F^+ - \Delta H^+)$, and $\Delta F^+ = RT\,2.303 \log (KT/hk)$; 1 mole $l.^{-1}$ was used as the standard state (Frost and Pearson, 1961). The errors given for ΔH^+ are at the 70% confidence level. Values of ΔH^+ and ΔS^+ are calculated at 25°.

[3] Coordination of imidazole to iron- or cobalt(III) complexes can have a pronounced effect on the ionization of the imino group and its heat of ionization (Hanania and Irvine, 1964; George *et al.*, 1964). The pK_a of this group, 14.5, in free imidazole falls to 10.3 in the imidazole–ferrimyoglobin or aquocobalamin complexes, while the enthalpy of ionization drops from 17.6 to 10–11 kcal $mole^{-1}$. These values are out of the range of the values of pK_a and enthalpies of ionization deter-

models. The data obtained here for pK_{EH} using CbzGlyGly-L-Leu and CbzGlyGly-L-Val as substrates (ΔH_i = 5.43 kcal mole^{-1} and ΔS_i = −23.5 eu) are very close to those known for phenol. For phenolic groups of dipeptides, the variation of ΔH_i is extremely small, regardless of whether a positive or negative side chain is vicinal to the phenolic group (Izatt and Christensen, 1968). Such values (6.0 ± 0.5 kcal mole^{-1}) differ significantly from those obtained for ε-amino groups (13 ± 0.5 kcal mole^{-1}).

The values of ΔH_i for pK_{EH_2} ($\simeq$3 kcal mole^{-1}) are intermediate between those obtained for carboxyl and imidazolyl groups of amino acids and peptides (Edsall and Wyman, 1958). However, when comparing the thermodynamic parameters determined for pK_{EH_2S} ($\Delta H_i \simeq 7$ kcal mole^{-1}; $\Delta S_i \simeq -6$ eu) to those for peptide models of protein side chains, these values would seem much more consistent with those of an imidazolyl group. In fact, they resemble closely the values obtained by Fife and Milstein (1967) for the deacylation of acyl chymotrypsin (ΔH_i = 7.0 kcal mole^{-1}; ΔS_i = −9.6 eu). In that instance, the evidence available through a number of approaches strongly favors the hypothesis that histidine is involved in the deacylation step for the chymotrypsin catalyzed hydrolysis of esters (Schoellman and Shaw, 1963; Crestfield *et al.*, 1963; Bender and Kezdy, 1964).

The pK_a values (*i.e.*, free energy of ionization) for carboxyl groups of aliphatic carboxylic acids can be increased by lowering the dielectric constant of the medium. The pK_a can be increased readily from values of 6–10 in a mixture of dioxane and water, but ΔH_i remains a small negative number while ΔS_i becomes increasingly more negative (Harned and Dedell, 1941). Thus, a hydrophobic environment in an enzyme might easily change the pK_a of a carboxyl group by 2 units, but this would likely be accomplished through a more negative ΔS_i rather than a more positive ΔH_i. In contrast the pK_a of positively charged acids such as imidazole have a slight solvent effect which is often negative (Findlay *et al.*, 1962; Lowe, 1970; Benkovic and Dunikoski, 1970).

In principle, environmental effects such as hydrophobicity of neighboring side chains or electrostatic effects could account for the different pK_a values in proteins and peptides. However, when pK_a values are determined kinetically, equilibria affecting dissociation of species other than the most important one could raise or lower the true pK_a quite as readily (Bruice and Benkovic, 1966). If such equilibria have a marked temperature dependence, the ΔH_i obtained by steady state kinetics will be a composite of the temperature dependencies of both the equilibrium constant and the ionization constant.

If such equilibria occur prior to the ionization, they will raise the pK_a. A strong hydrogen bond between the protonated form of a carboxyl group and some other group which does not ionize in the region of pK_{EH_2} or pK_{EH_2S} could be cited as an example of such an equilibrium. However, this equilibrium constant must have a large temperature dependence in order to exhibit an apparent ΔH_i similar to that of a histidyl residue.

If such equilibria occur subsequent to the ionization, they will lower the pK apparent (Bruice and Schmir, 1959). The participation of the imidazole group in the hydrolysis of phenyl esters of γ-(4-imidazolyl)butyric acid exemplifies the situation where the kinetically determined pK_a differs from the true pK_a of the participating group owing to equilibria which occur after ionization but prior to the rate-determining step (Bruice and Sturtevant, 1959). Piszkiewicz and Bruice (1968) have also suggested the occurrence of such a mechanism in the lysozyme-catalyzed hydrolysis of *p*-nitrophenyl acetate derivatives. The unprotonated His-15 was suggested to be the active species, but, when unprotonated, it could also interact with threonine-89 causing an effective lowering of the apparent pK_a of the histidyl residue to a value of 5.2.

In the present instance yet other alternatives could be considered. Two of the zinc ligands in carboxypeptidase A have been identified to be histidyl residues (Bradshaw *et al.*, 1969; Lipscomb *et al.*, 1969). Hence, competition between protons and the metal for one of their imidazolyl groups might be able to inactivate the enzyme. In such an instance, pK_a' would indeed be expected to be lower than the true pK_a of a histidyl residue. If the temperature dependence of the association constant for this ligand–metal interaction is negligible, changes in pK_a' as a function of temperature might still be a measure of the enthalpy of ionization of this histidyl residue. However, the verification of such an equilibrium by direct observation would present experimental problems. A suitable method would have to be capable either of detecting different properties of the residue in its two extreme equilibrium states or of rapidly trapping the intermediate. Conventional chemical modifications would not seem suitable, since predominantly the residue providing the ligand would likely exist in one of two inactive states, *e.g.*, either as an imidazolium ion or bound to the metal. Theoretically it might be possible to detect such an intermediate under conditions analogous to those which have proven effective in studying conformational changes of aspartic aminotransferase during catalysis (Christen and Riordan, 1970).

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Mechanism of Action of Uncouplers of Oxidative Phosphorylation*

David F. Wilson,† H. P. Ting,‡ and Michele S. Koppelman

ABSTRACT: Four chemical types of uncouplers of oxidative phosphorylation in rat liver mitochondria have been studied with respect to the pH dependence of their uncoupling activity.

These uncouplers include salicylanilides, carbonyl cyanides, halogenated benzimidazoles, and thiophenols. The uncoupling activity in each case is pH dependent and, when the p*K*'s of the dissociable groups and solubilities in aqueous media are considered, this pH dependence is consistent with the uncouplers acting as acid or base catalysts of a reaction occurring in the nonaqueous region of the mitochondrial membrane. The carbonyl cyanide derivatives and halogenated benzimidazoles appear to be active as acids (protonated form) while the salicylanilides and halogenated thiophenol appear to be active as bases (anionic form). The measured pH dependences of the uncoupling activities do not support hypotheses in which the uncouplers act as proton carriers or as transported anions.

The compounds which uncouple the energy conservation reactions from the oxidation–reduction reactions of mitochondria (as opposed to those which activate ion transport, such as valinomycin, etc.) were first thought to act by chemical mechanisms (Loomis and Lipman, 1948; Slater, 1953; Lardy and Wellman, 1953; Chance and Williams, 1956). In recent years, however, the proposed mechanisms have been expanded to include additional chemical mechanisms (Hemker, 1964a,b; Weinbach and Garbus, 1969; Wang, 1967), proton conduction mechanisms (Mitchell, 1961, 1966), and transported anion mechanisms (van Dam and Slater, 1967).

The uncoupler molecule, in general, is characterized by having a chemical group from which a proton is dissociable with a p*K* between 3 and 9 (DeDeken, 1955; Parker, 1958). In addition a solubility in organic solvents seems to be important (DeDeken, 1955; Hemker, 1964a,b).

In the present work we have attempted to determine the role of the dissociable proton in uncoupling. For this purpose

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† To whom to address correspondence.
‡ Present address: Department of Biochemistry, Michigan State University, East Lansing, Mich.