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Chemical Modification in the Active Site of δ-Chymotrypsin

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The properties of the active site of δ-chymotrypsin were studied by specific modification of two side chains with dimethyl diimidates of aliphatic dicarboxylic acids. The sites of modification were identified as His-57 and Met-192 using ^{14}C-labelled reagent and amino acid sequence analysis of the NH-terminal portion of modified peptides. The latter were liberated from the modified enzyme by limited proteolysis and isolated. The active site titer remained unity after the modification, but the substrate specificity had changed in favour of specific esters. In contrast, the specific amidase activity was diminished. Treatment of derivatives of His and Met with the reagent confirmed the feasibility of the modification reaction proposed. The optical characteristics of the modified enzyme (MCT) support the chemical evidence for the nature of the modification.

Chemical modification in the active site of an enzyme is one of the most direct approaches to study the catalytic mechanism. Covalent derivatives of functional groups which in the native state of the enzyme participate in the conversion of the substrate are almost invariably inactive, or nearly so, but a few exceptions exist.[1,2] One example is reported here. The retention of the catalytic power by the derivative was probably due to enhancement by the modification of the very property, *e.g.* nucleophilicity or basicity, which represented the normal action of the functional group. The specificity, however, was, in the case of chymotrypsin (CT),* sharpened and shifted in the direction from amidase toward esterase activity.[3]

Initially, the project was designed to anchor a group, the NH-terminus of the B-chain, which was expected to participate in the stabilization of the active site by electrostatic clamping of the Asp-194 carboxylate group. The latter side chain would otherwise rotate into the polar environment of the active site and more or less directly impede the productive binding of the substrate. An amidination of Ile-16 would, if it had been successful, have

* *Abbreviations:* ATEE, *N*-acetyl-L-tyrosine ethyl ester; CT, Chymotrypsin; DNS, 1-Dimethylamino-naphthalene-5-sulfonamide; MCT, CT modified by dimethyl malondiimidate; NMR, Nuclear magnetic resonance; ORD, Optical rotatory dispersion; PTH, Phenyl-thiohydantoin; RNase, Ribonuclease; Tris, Tris(hydroxymethyl)aminomethane.

shifted the pK-value of the α-amino group sufficiently in alkaline direction to keep the group almost fully charged under physiological conditions and hence fulfil the intended mission. However, this NH-terminus proved to be much less reactive (only 22 % of the value to be expected from the Brønsted-plot)[4] than normally. This has recently been independently confirmed.[44] Instead of NH-modification, a derivative of high, specific esterase activity and low, specific amidase activity appeared. Its characterization is described below.

Since selectivity of the modification reaction is essential if the observations are intended for mechanistic interpretation, the derivatization was commenced by reversible or irreversible masking of potentially competing groups, such as the ε-amino groups. The latter are in the case of CT known to be unimportant to the catalysis of the native enzyme and should, therefore, be prevented from interfering with the intended, specific modification of the active site.[3] The ε-amino groups were maleylated or carbamylated in the zymogen where the active site is protected (see Fig. 1). Full activity appeared upon activation of the masked zymogen by the conventional procedure.

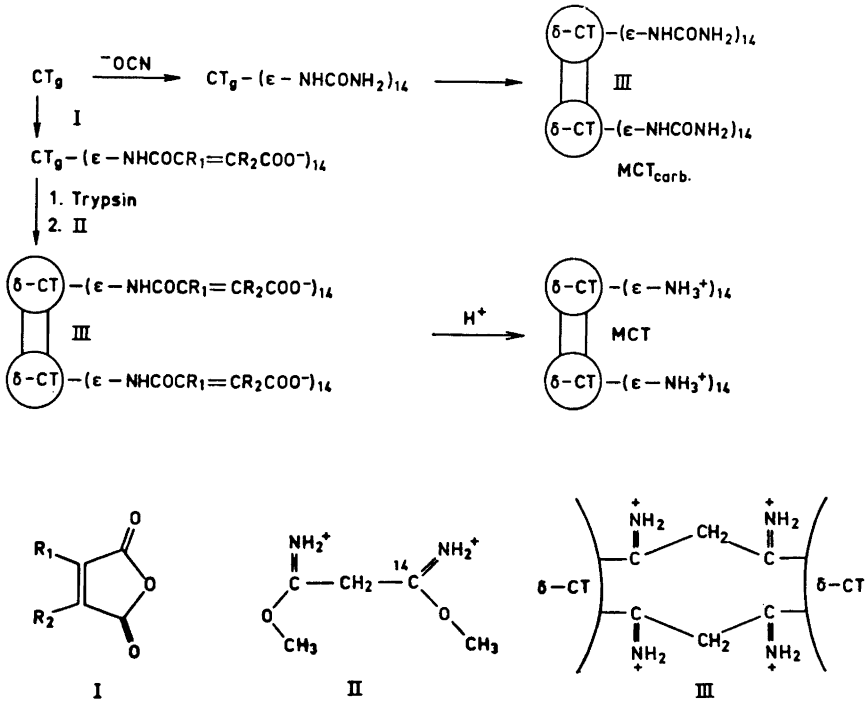


Fig. 1. Summary of the procedure for preparation of MCT. Two alternative pathways are shown. Native chymotrypsinogen is used in both. The two methods of protective masking of competing groups are used for different purposes: Carbamylated MCT is used for quantitative isotope studies whereas transient maleylation is used for crystallization of MCT, for X-ray diffraction work, sequenation, kinetics, and optical measurements. (See also text).

A bifunctional, rather than a monofunctional, reagent was initially chosen because the aim of the project then was to anchor a group (α-NH_2 of Ile-16) by a short bridge. Besides, exploratory experiments had indicated that monofunctional imidates had no effect on the activity of CT.[6] Dimethyl diimidates of short chain, aliphatic dicarboxylic acids, however, had a marked effect on the catalytic efficiency and specificity.

Imidates are useful reagents because they normally are specific for amino groups and react under mild conditions but they are difficult to prepare in pure state. However, the impurities, which normally consist of small amounts of carboxylic acids, nitriles and ammonium salts, do not interfere with the intended reaction.

Most modification reactions designed to elucidate enzyme mechanisms employ reagents which per molecule attack a single site, but in a few instances, bifunctional reagents have been used.[7–11] In this case, a short chain was preferred to favour a rigid fixation of the reacting groups. This resulted in a stabilization of the active site and in a rate enhancement which increased with decreasing bridge length.[3]

Table 1. NMR-Resonance of histidine and malonic acid derivatives. Comparison between the NMR spectra of compounds closely related to dimethyl malondiimidate and the reaction product of this compound with *N*-acetyl-L-His. Operating frequency: 60 MHz. Sweep times: 50 and 500 sec. R. F. field: 0.02 and 0.04 mG. Temp. 23°. Solvent = D_2O.

Compound	δ[a] ppm	$\Delta\delta$[f]	Multiplicity	H[b]	Assignment	Area ratio
Imidazol	7.22		Douplet $J=1$[c]	3	$\geq$C–H	10
	7.85		Singlet[d]			7
L-His	7.08		Douplet $J=1$	2	$\geq$C–H	1
	7.79		Douplet $J=1$			1
N-Ac-L-His	7.49		2 Douplets $J=1.5$ and 5	2	$\geq$C–H	1
	8.49		2 Douplets $J=1.5$ and 8			1
His-der.[g]	7.37	0.12	Douplet $J=1.5$	2	$\geq$C–H	1
	8.20	0.29	Douplet $J=2.5$			1
Dimethyl malon-	3.82		Singlet[d]	6	$-OCH_3$ and	
diimidate	3.82		Multiplet	2	$-CH_2-$	
Malonoditril[h]	7.58		Triplet	2	$-CH_2-$	1 : 2 : 1
Malonic acid	3.54[e]		Singlet[d]	2	$-CH_2-$	
L-His	3.18		Multiplet	2	$-CH_2-$	2.0
	4.00		Triplet	1	α-H	1.0(1 : 2 : 1)
N-Ac-L-His	2.07		Singlet[d]	3	$-COCH_3$	9
	2.13		Singlet	2	$-CH_2-$	6
	3.40		Triplet	1	α-H	*Ca.* 1 : 2 : 1
His-der.[g]	1.92	0.15	Singlet[d]	6	$-COCH_3-$	1
	1.95	0.18	Douplet $J=5$	6	$-CH_2-$	1
	3.00	0.40	Multiplet	2	α-H	

[a] Chemical shift. Standard: Tetramethylsilan. [b] Number of hydrogen atoms involved. [c] Two overlapping doublets + 1 singlet. J = coupling factor in cps. [d] Sharp peak. [e] The peak vanishes due to D–H exchange. Half time = 1 min. [f] (δN-*Ac*-L-*His* – δ*His-der*). [g] His-der.: Product of the reaction of *N*-acetyl-L-His with dimethyl malondiimidate. [h] Solvent: DMS.

METHODS

Preparation of the reagent. Dimethyl malonic- and succindiimidates were prepared by the method of Hunter and Ludwig [12] with the following modification: The corresponding dinitrile was dissolved in methanol instead of ether or chloroform. The reaction product was identified by determination of the melting point (about 98°), by measurement of IR, NMR, and mass spectra and by elemental analysis. The IR-spectrum displayed the ester absorption band and the UV-spectrum the imidoester chromophore. The NMR-spectrum showed the expected resonances (see Table 1) and the fragmentation pattern obtained by mass spectrometry was compatible with the assumed structure.

A radioactive label (^{14}C) was introduced in the carboxyl groups.[13,14] The specific radioactivity of the compound was determined by scintillation counting using Bray's solution and calibration with standard ^{14}C-toluene.

Treatment of N-*acetyl*-L-*Met by dimethyl malondiimidate.* The purpose of this experiment was to prove that the Met side-chain could react with the diimidate. The procedure which was employed followed the one for MCT (see below) as closely as possible. Example of an experiment:

Three mg of *N*-acetyl-L-Met were dissolved in 20 ml of an 0.1 M Tris – HCl buffer at pH 8.0. An 0.2 ml aliquot of this stock solution was diluted to 2.00 ml with the buffer in the cuvette at 25° and the reaction was started by addition of 1.0 mg of dimethyl malondiimidate and mixing. Full UV-spectra were recorded immediately and repeated every 10 min. The reaction product was characterized by spectrophotometry since it was so labile that it was difficult to isolate.

Treatment of N-*acetyl*-L-*His by dimethyl malondiimidate.* This experiment is analogous to the one above. In a typical experiment 5 mg of *N*-acetyl-L-His were dissolved in 10 ml of an 0.1 M Tris buffer at pH 8.1. An aliquot of 3.00 ml was placed in a cuvette and the reaction was initiated by addition of 2.5 mg of dimethyl malondiimidate. Complete UV-spectra were recorded until near completion of the reaction. Since Beer's law was not obeyed, the concentration dependence of the extinction coefficient was determined by serial dilution. Extrapolation to infinite dilution and to saturation yielded limiting values which were used for the determination of the prototropic dissociation constants after repetition of the experiment at a number of pH-values.

The amidine chromophores at 1580 cm^{-1} and at about 240 nm were observed by IR- and UV-spectrophotometry.[15] The content of C, H, and N was determined by microanalysis and the molecular weight was calculated from the colligative properties. The structure was also studied by mass spectrometry.

Masking of ε-amino groups in chymotrypsinogen. (a) Reversible masking. The procedure was essentially that described earlier by Harris, Perham *et al.*[16,17] In a typical experiment 50 mg of the zymogen was dissolved in 10 ml of an 0.05 M phosphate buffer at pH 7.5 and 4°. Dimethyl maleic anhydride was then added in a 50-fold molar excess. The progress of the reaction was followed in a pH-stat. After near completion of the hydrolysis of excess reagent, the by-products were removed by dialysis at pH 5.0.

(b) Irreversible masking. The cyanate method of Stark [18] was used for this step. Fifty mg of the zymogen was dissolved in 10 ml of an 0.02 M borate buffer at pH 8.0. The buffer contained 0.2 M KOCN and 8 M urea. The reaction was allowed to proceed at room temperature for 1 h. Then, the solution was dialyzed.

Activation of chymotrypsinogen with masked ε-amino groups. The modified zymogen was activated by a brief exposure to trypsin according to the procedure of Jacobsen.[5] The activity gain and the detection by the DNS-method (see below) of Ile (but not of Ala) as NH-terminus proved that the reaction had taken the desired course.

The modification reaction. The modification was performed at pH 9.7 and 7° in the presence of a large excess (400-fold) of specific substrate (ATEE) to ensure that the enzyme would become locked in an active and not in an inactive conformation. The reagent, dimethyl malondiimidate was added to the zymogen (in which the ε-NH groups had been masked) in 2000-fold excess in two equal batches, the second 10 min after the first. The progress of the reaction was followed with a pH-stat. The reaction was complete in 30 – 60 min. Excess reagent was hydrolyzed and all by-products were removed by exhaustive dialysis at pH 3 and 4 for 25 h with 4 changes (each 4 l) of HCl solution. Usually, 50 – 100 mg of chymotrypsinogen was used for preparation of a batch of modified enzyme The dialyzed solution of this enzyme (MCT) was distributed in small plastic vials and

frozen. In the frozen state the compound was stable for several months since the UV-spectrum and the specific esterolytic activity (toward ATEE) remained unchanged. The entire procedure for preparation of MCT is summarized in Fig. 1.

Measurement of the incorporation of ^{14}C-reagent into the enzyme. The concentration of MCT on a monomer basis was determined from the absorption at 280 nm using the extinction coefficient which was obtained by correlation of the Kjeldahl analysis with the UV-spectrum.[3] The specific ^{14}C-activity of MCT was measured on a low-level counter (background: 2 – 4 cpm). Measured samples of MCT were pipetted into shallow trays (15 cm ϕ, Al) and dried under infrared light before counting. From this information, the number of equivalents of ^{14}C-labelled reagent incorporated in each CT-monomer could be calculated with a relative error of a few percent.[19]

Determination of the operational active site titer of MCT and CT. The spectrophotometric cinnamoyl imidazole titration method of Schonbaum *et al.*[20] was used. The relative error involved was a few percent. The cinnamoyl imidazole reagent was prepared by the method of Schonbaum *et al.*[20] and recrystallized. Its melting point and UV-visible spectrum were checked.

Identification of the modified sites. (a) Amino acid sequence analysis. A sample of MCT was treated with an excess of dithiothreitol by the method of Cleland [21] to cleave the disulfide linkages reductively. Subsequently, the sulfhydryl groups were completely masked by alkylation with an excess of iodoacetate in 4 M urea.[21] The A-chain was removed by exhaustive dialysis at pH 3.[25] The remaining chains were subjected to sequential, limited proteolysis at 25° in the pH-stat by trypsin [22] at pH 8 (for 20 h) and thermolysin (for 6 h at 40° and pH 7.5).[23,24] The resulting peptides were fractionated by three, consecutive column chromatographic steps:[25]

Gel filtration: Sephadex G-25 in 0.05 M phosphate at pH 7.5 and 4° (72 × 2.7 cm).

Ion exchange: Dowex 50 at 4° and pH 3.1 – 5.6 (gradient) in pyridine – acetate (72 × 2.7 cm).

Gel filtration: Sephadex G-10 at 4° and pH 7.5 in 0.05 M phosphate, 8 M urea (48 × 2.7 cm).

The last step was repeated until the fractionation appeared to be complete. The elution profile for ^{14}C was in each step used to locate the labelled peptides. Four radioactive peptides (D_1, D_2, E_{1a}, and E_{1b}) representing 90 % of the radioactivity were sequenced. All the four peptides displayed the amidine chromophore at about 250 nm. Their homogeneity was tested after rechromatography on Sephadex G-10 or Dowex 50 by high voltage paper electrophoresis at pH 6.5 and 10° for 30 min. Since it was found to be satisfactory, amino acid sequence analysis was performed on the four peptides by the subtractive method of Hartley and Grey.[26]

Polyamide sheets were used to separate the DNS-amino acids.[27] The phenylthiohydantoins were collected at each stage and their radioactivity was measured. Samples of each peptide were subjected to hydrolysis for 20 h at 110° in 6 N HCl and the amino acid composition was determined by the ninhydrin method after ion exchange chromatography.

The following solvents were used for the chromatography of DNS-amino acids on polyamide sheets.

In the first direction: Formic acid in water (1.5 % v/v). In the second direction: Benzene – acetic acid (9:1, v/v).

After drying, again in the 2nd direction: Ethyl acetate – methanol – acetic acid (20:1:1, v/v).

Standards: DNS-Gly, DNS-Glu, DNS-Ile, DNS-Phe, DNS-Pro, DNS-Ser, and DNS-Arg.

The standards were applied on one side of the sheet, the sample on the opposite. A small portion of the sample was also applied to the reference side. In this way, the conditions for chromatography of the unknown sample and the reference became identical. The sample spots on the reference side of the sheet could be distinguished from the reference spots by the difference in the intensity of the fluorescence.

(b) Selective performic acid oxidation of Met-192 prior to the reaction with diimidate. The method of Weiner *et al.*[28] was used to destroy a suspected site of modification. In the subsequent attempt to modify the enzyme by the diimidate reaction according to the procedure described above, the process failed to take the usual course.

Estimation of the molecular weight of MCT. Two different methods, gel filtration at

4° on calibrated columns and analytical ultracentrifugation, were employed for this purpose. In the former, Sephadex G-100 (medium) was used in an 0.05 M phosphate buffer at pH 7.5 with and without 8 M urea. The low temperature was chosen to reduce autolysis and uncontrolled denaturation. The columns were calibrated with chymotrypsinogen, pepsin, β-lactoglobulin, pepsinogen, hemoglobin, fibrinogen, and blue dextran.

Since the gel filtration method requires that the proteins have a spherical shape, the experiment was repeated in a denaturing solvent (8 M urea), which is believed to favour this shape since it weakens the intramolecular, non-covalent interactions.[29] In addition, this solvent served to cleave possible non-covalent aggregates of MCT. The compounds were identified by their individual UV- or visible spectra and the elution volumes were used to compute the parameter, K_{av}, which resembles the partition coefficient. This parameter served to define the calibration curve for estimation of the molecular weight.[30]

Analytical ultracentrifugation was applied to check the estimate of the molecular weight of MCT. This was performed in buffer solution at 25° by the Archibald approach to equilibrium method and by combination of sedimentation velocity and boundary spreading data. The molecular weight of α-CT was also determined to check the methods. The agreement with known data was good.

Other physical measurements. The protein concentration was determined by measurement of the extinction at 280 nm.[3] Infrared spectra were recorded on a Beckman instrument. The NMR-spectra were measured on a Varian instrument (60 MHz). Elemental analysis and determination of the molecular weight of the reaction product of *N*-acetyl-L-His with dimethyl malondiimidate were performed by Beller Micro-analytical Laboratory, Göttingen. Optical rotatory dispersion was recorded on a Spectropol 1 spectropolarimeter from FICA. The active site titrations of the operational normality of the enzyme [19] were performed on a Cary-14 spectrophotometer.

Materials. The chymotrypsinogen was a 6-times crystallized product from British Drug Houses, α-chymotrypsin a crystalline preparation (activity: 1100 NG/mg) from NOVO Industries, Copenhagen, and the marker proteins, crystalline products from Miles-Seravac, Worthington and Boehringer. Chymotrypsin was purified from a contaminating peptide by gel filtration according to the method of Yapel *et al.*[32] The organic compounds used for the syntheses were analytical grade reagents from Fluka, British Drug Houses, and Koch & Light. The solvents were dried and distilled. The polyamide sheets were purchased from Cheng Chin Trading Co., Hankow St., Taipei, Taiwan. Radio active NaCN (^{14}C) was purchased from the Radiochemical Center, Amersham, England. Its specific activity was 52.8 mC/mmol. Standard ^{14}C-toluene was acquired from the same source.

RESULTS

The reactivity of methionyl and histidyl side chains toward dimethyl malondiimidate. The rate constants for the modification of *N*-acetyl-L-Met were determined by recording the absorbance as a time function at a band, 266.7 nm, which is characteristic of the modified Met side chain. The spectrum of the Met derivative is shown in Fig. 3. The kinetics of its formation were analyzed as described in the legend of Fig. 4. The corresponding plot is shown in Fig. 4. The maximal absorbance change under the conditions of this experiment was 0.360 which corresponds to a difference extinction coefficient of 1432 l mol^{-1} cm^{-1}. The rate constant for the modification of Met is compared with that of similar reactions in Table 3. Table 3 shows that both *N*-Ac-L-Met and *N*-Ac-L-His can react with dimethyl diimidate and that the 2nd order rate constants are of the same magnitude as those found for similar imidates when they react with amino groups.

The stability of the Met derivative was studied at 255.8 nm (39.1 kc). At pH 7.0 and 25°, the reaction follows pseudo first order kinetics.

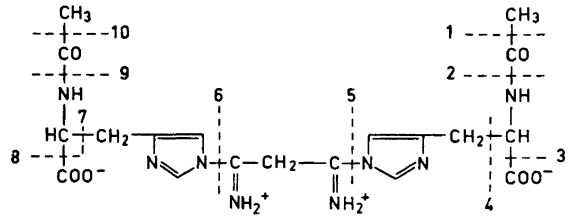


Fig. 2. The suggested structure of the reaction product of the treatment of *N*-acetyl-L-His with dimethyl malondiimidate. The numbers indicate the fragmentation points by mass spectrometry which are expected from comparison with the decomposition of similar compounds.

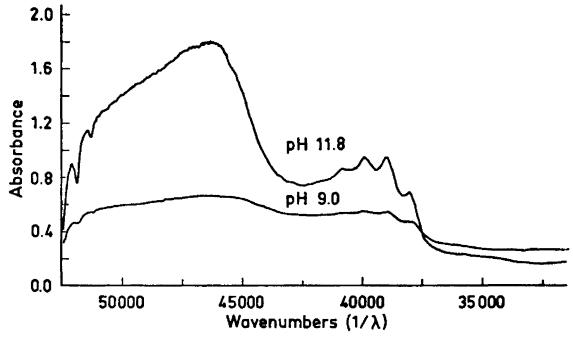


Fig. 3. The UV-spectrum of the product of the reaction of *N*-acetyl-L-Met with dimethyl malondiimidate (2.5×10^{-4} M in H_2O at 25°). Reference: H_2O. Light path: 1 cm.

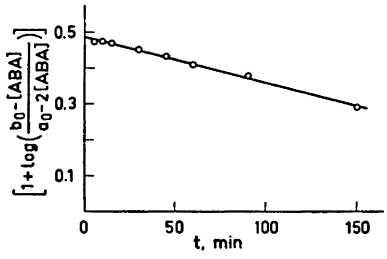
Fig. 4. The kinetics of the reaction of *N*-acetyl-L-Met with dimethyl malondiimidate at pH 8.0 and 25° in 0.1 M Tris – HCl measured at 266.7 nm. None of the reactants have an absorption band in that range. Assuming the reaction

$$2A + B \xrightarrow{k_1} AB + A \xrightarrow{k_2} ABA,$$

additivity of the absorption contributions from the two chromophores: $\varepsilon_{AB} = 1/2\ \varepsilon_{ABA}$ and that AB is in the steady state, the parameter $k_1k_2/(k_2 - k_1) = (2.30 \pm 0.02) \times 10^{-2}$ l mol^{-1} sec^{-1} may be evaluated from the slope

$[= \{(2\ b_0 - a_0)k_1k_2/(k_2 - k_1)\} \times 0.868]$.

Symbols: a_0 = initial conc. of *N*-Ac-L-Met = $= 7.85 \times 10^{-4}$ M; b_0 = initial conc. of dimethyl malondiimidate = 2.48×10^{-4} M.



The modified Met displayed in the alkaline range a characteristic 5-member absorption band resembling that of benzene. This spectrum could be directly observed in the modified enzyme under the same conditions (see Fig. 5). The spectrophotometric titration curve for one of these bands (the one at 250.2 nm) was determined because the pK-value of the corresponding function had to be taken into account by the interpretation of changes in the pH-dependence of the kinetic parameters upon conversion of δ-CT to MCT and for studies of the ionization properties of the Tyr side chains in the modified enzyme.

The His derivative was subjected to a similar analysis. Its formation was too fast to be followed with the equipment available. However, the compound was stable enough to be isolated by crystallization and was subsequently studied by mass spectrometry, UV-spectrophotometry (see Fig. 6), elemental analysis (Found: C 47.1; H 6.4; N 6.1. Calc. for His der.: C 46.7; H 5.6; N 12.1.

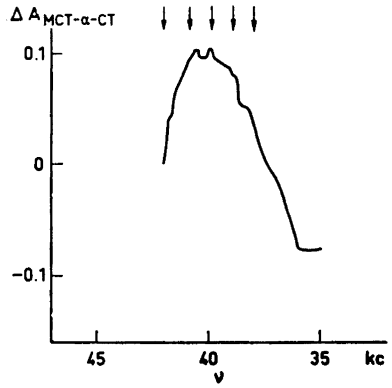


Fig. 5. Difference spectrum between MCT and α-CT at pH 9.8 and 23°. The chromophore of the modified Met is seen superimposed upon the band of the modified His (see Fig. 6) and those of the perturbed Trp and Tyr residues. The arrows show the position of the bands in the model compound (see Fig. 3). Conc.: MCT = α-CT = 10^{-5} M; $\nu = 1/\lambda$ (wavenumber).

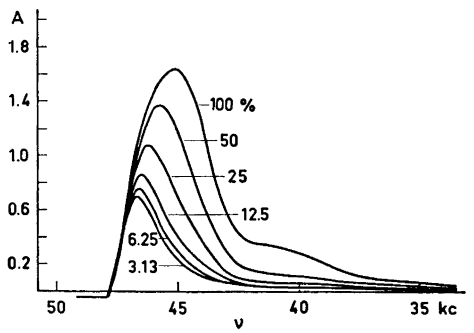
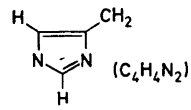


Fig. 6. Spectra of the product of the reaction of *N*-Ac-L-His with dimethyl malondiimidate (2.54 and 12.4 mM, respectively) at pH 9.7 in 0.1 M Tris. The spectra were recorded at pH 10.1 *vs.* 0.1 M Tris (1 cm light path, 23°) after completion of the reaction and the following serial dilution: 100, 50, 25, 12.5, 6.25, and 3.13 % of original conc. Ordinate: Absorption. Abscissa: Wavenumber ($1/\lambda$).

Calc. for N-Ac-His: C 48.5; H 6.1; N 21.2) and molecular weight determination (Found: 440; Calc. for His der.: 462. Calc. for N-Ac-His: 198). The fragmentation pattern was analyzed on the basis of the hypothetical structure [33,34] which is shown in Fig. 2. The results are presented in Table 2.

The first order rate constant for hydrolysis at pH 9.7 and 4° of dimethyl malondiimidate ($k = 0.136$ min^{-1}) was taken as a measure of the reactivity of the compound. This value may be compared with 0.018 min^{-1} at pH 9.0 and 25° and 0.024 min^{-1} at pH 10.6 and 25° for methyl acetimidate [12] and 0.00033

Table 2. Fragmentation pattern of the His derivative. Mass spectrum of the product of reaction of *N*-acetyl-L-His with dimethyl malondiimidate. Conditions: 300°, 1600 V, 100 μA. Symbols: M, The assumed structure (intact) as shown in Fig. 2; i, M cleaved at point 5 and/or 6 ($C_8H_9N_3O_3$); B: $C(=NH_2^+)-CH_2-C(=NH_2^+)$ (bridge); I:



m/e Obs.	*m/e* Calc.	Composition	Cleavage points	Fragments	Components
501	501	$C_{17}H_{18}N_8O_6Cl_2$	1, 10	MCl_2-2CH_3	
372	372	$C_{17}H_{24}N_8O_2$	3, 8	$M-2\ CO_2$	
266	266	$C_{11}H_{14}N_6Cl$	4, 7	$M-2CHNH(CO)CH_3$ (with CO_2^-)	
256	256	$C_{13}H_{16}N_6$	3, 8		B + 2 I + 2 CH
237	237	$C_{10}H_{13}N_4O_3$			i + $CH_2-C=NH_2^+$
180	180	$C_7H_6N_3O_3$	1, 5, 6, 10	$i-CH_3$	
151	151	$C_7H_9N_3O$	3, 5	$i-CO_2$	

Table 3. Rate constants of product formation. Comparison between the reactivities (k) of various groups in amino acids towards imidates.

Amino acid or peptide	Imidate	Temp. °C	pH	k l mol^{-1} min^{-1}	Product
ε-NH_2-caproic acid	Methyl benzimidate	25	8	2.2[a]	Amidine
Gly–Gly	Methyl benzimidate	25	8	4.2[a]	Amidine
None	Methyl benzimidate	39	8.5	< 0.0001	Benzoic acid
N-Ac-L-Met	Dimethyl malondiimidate	25	9.7	1.4	Di-thioimidate[b]
N-Ac-L-His	Dimethyl malondiimidate	25	9.7	> 15	Amidine[b]
N-Ac-L-His	Methyl acetimidate	25	7.4	0	Amidine[b]
None	Methyl acetimidate	25	7.4	0.0003	Acetic acid.

[a] Ref. 12. [b] Suggested on the basis of spectral and titrimetric evidence (see text).

min^{-1} for methyl benzimidate[12] at pH 9.6 and 39°. The malondiimidate is clearly much more reactive than the previously used monofunctional imidates.

The spectrophotometric titration curve for modified *N*-acetyl-L-His was difficult to obtain because this compound undergoes reversible oligomerization. Therefore, the spectrum of the monomer had to be constructed from the spectra of the substance at 5–6 different concentrations (serial dilution) at each

pH-value (see Fig. 6). The data listed in Table 4 are based on the Linderstrøm-Lang theory[35] and on the reported pK'-values, pK_{intr}-values, electrostatic parameters, and difference extinction coefficients per prototropic group.[31]

Table 4. Protolytic and spectroscopic parameters of enzymes, derivatives and model compounds. The data in this table were collected at room temperature (*ca.* 23°) unless otherwise stated. They serve to aid the identification of sites of chemical modification or changed environment and the characterization of the nature of such alterations (*e.g.* electrostatic field, polarity of environment or conformational changes).

Compound	pK'	pK'_{intr}	h	w	$\Delta\varepsilon^{294}$ l mol^{-1} cm^{-1}	Tentative assignment
His-der [a]	9.43	7.48	1.85	0.90		ring 1
	10.10					ring 2
	10.40					amidine 1
	10.76					amidine 2
	11.55	8.94		1.21		amidine 3
Met-der [b]	9.29 ± 0.09	8.87 ± 0.08	1.26 ± 0.01	0.42		imido 1
	12.2					imido 2
MCT [c]	6.32		4.97		160 000	His 1
8 M urea	7.41		3.08		16 000	His 2
24°	8.56		8.56		14 000	His 3
	9.82 ± 0.03					
	10.25					
	11.00 ± 0.02	9.92 ± 0.03	0.60 ± 0.04	0.17 ± 0.01		
α-CT[c]	7.02	5.29	2.20	0.67		2 His
25°	8.15					2 $-NH_2$
0.15 M KCl	6.20					1 COO^-
	5.75					1 COO^-

[a] The product of the reaction of *N*-acetyl-L-His with dimethyl malondiimidate. [b] The product of the reaction of *N*-acetyl-L-Met with dimethyl malondiimidate. [c] Protolytic titration. Ref. 42. h Slope of log A/HA *vs.* pH-function. w Electrostatic factor (see text). $\Delta\varepsilon^{294}$ Difference extinction coefficient at 294 nm.

NMR-spectra of derivatives of His and malonic acid were recorded. The resonance frequencies, multiplicities, and area ratios are listed in Table 1. These data are in agreement with the values reported in the literature for similar compounds. The treatment of *N*-acetyl-L-His with dimethyl malondiimidate gives rise to a shielding effect of the resonances of the protons at the ring, the α-C, the β-C, and the acetyl group. This diamagnetic shift is more pronounced for the His side chain than for the acetyl group.

Characterization of the modified enzyme. The elution profile, gel electrophoresis at pH 6.5 and determination of the number of NH-termini were used as criteria for the purity of the preparation. The curves for the determination of the molecular weight by gel filtration were illustrated in Fig. 1 of a preliminary communication.[3] In the same article, the data for the extinction coefficients at 280 nm and the number of amide and free amino groups in α-CT, dimethyl maleylated δ-CT, and carbamylated δ-CT were given in Table 1.

In addition, the extinction coefficient at 280 nm for δ-CT modified by dimethyl succindiimidate was listed. The latter was prepared by a method analogous to the one described in this article for dimethyl malondiimidate.

The results of the cinnamoyl imidazole titration of the operational titer of active sites in α-CT and MCT are shown in Table 5 which also contains the value for the incorporation ratio of carboxyl-^{14}C-labelled dimethyl malondiimidate into MCT.

Table 5. Comparison between the covalent dimer and the monomer. Evidence for the molecularity of the modification reaction and the accessibility of the active site in MCT for a specific substrate. The molecular weight was estimated by gel filtration on calibrated columns with and without denaturing agents (8 M urea) and checked by hydrodynamic measurements. The active site titer (= operational normality of the enzyme) was determined with *trans*-cinnamoylimidazole. The number of reagent molecules introduced per monomer was assayed with ^{14}C-dimethyl malondiimidate (carboxyl-labelled). The absence of sulfhydryl groups after the reaction was confirmed with Ellman's reagent.

Enzyme	M	Active sites/monomer eq./mol	^{14}C/mon. g-atom/mol	SH/monomer eq./mol
CT-monomer	25000	1.00 ± 0.03[a]		0.00 ± 0.02
M-δ-CT	47000 ± 3000	1.03 ± 0.03	1.030 ± 0.03	0.10 ± 0.02

[a] This value was 0.91 ± 0.03 before gel filtration.

Investigation of the possibility of disulfide cleavage in MCT during the modification reaction. Native chymotrypsin does not contain any sulfhydryl groups, but it was conceivable that imidates were sufficiently nucleophilic to break disulfide linkages, *e.g.* followed by the formation of a thioimidate with the reagent (dimethyl malondiimidate). The most reactive of these sites is the one involving Cys-191, the nearest neighbour to one of the expected sites of reaction.[35] An Ellman titration showed that this did not occur to any appreciable extent (see Table 5).[37]

Fractionation of peptides from MCT after disulfide cleavage and limited proteolysis by trypsin and thermolysin. The elution profile of two of the three chromatographic steps are shown in Figs. 7 – 8. The fractions which were taken for further purification are shown on the figures. Each fraction was pooled and concentrated by ultrafiltration before application to the next column. The gradient was chosen empirically. It was generated by a gradient former with two cylindrical vessels containing pyridine-glacial acetic acid buffers: 1. pH 3.1 and 5.0; 2. pH 5.0 and 5.6; 3. pH 5.6.[38] The fractions A – F were separately evaporated to dryness on Al-plates (15 cm ∅), weighed and counted. The radioactivity was related to the number of peptide linkages. The latter, which was taken as a measure of the length of the peptide, was estimated from the extinction at 230 nm which largely is due to this chromophore since the peptides are in the random coil conformation and since the content of aromatic side chains is low.

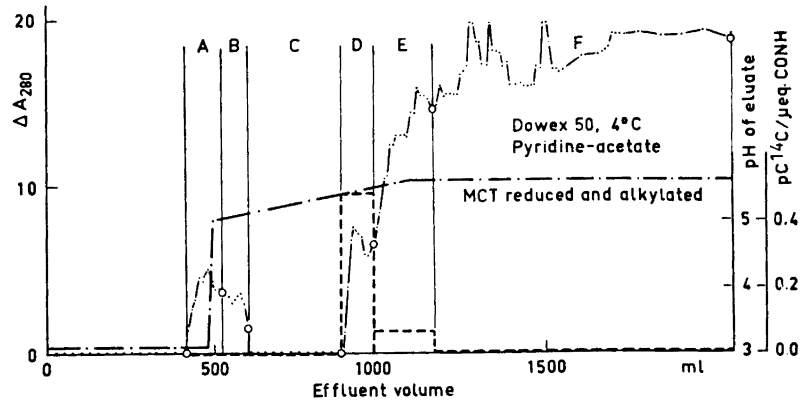


Fig. 7. Elution profile at 4° of peptides from limited proteolysis of carbamylated, reduced, alkylated M-δ-CT by trypsin and thermolysin. The column consisted of Dowex 50 × 2 ion exchange resin in equilibrium with pyridine-glacial acetic acid at pH 3.1. The buffer was a mixture of pyridine and glacial acetic acid (see text). Elution rate: 10 ml/h. Symbols: ———, absorption at 280 nm; —— · ——, pH of the buffer gradient; — — —, the specific ^{14}C-activity in cpm per equiv. of peptide linkage. The fractions A-E were separately collected. Fractions D and E, which contained all of the ^{14}C-label, were further fractionated on Sephadex G-10.

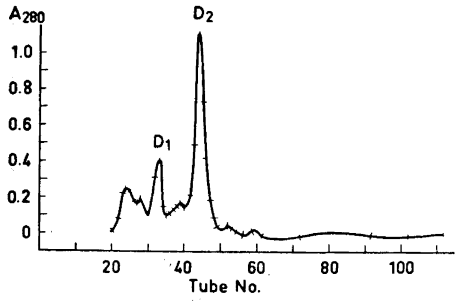


Fig. 8. Elution diagram showing the fractionation of fraction D (see Fig. 7). on Sephadex G-10 in 0.05 M phosphate, 8 M urea at 5° and pH 7.5. Flow rate: 10 ml/h. Column 57 × 2.7 cm. Ordinate: Absorption at 280 nm. Abscissa: Glass number (*ca.* 6.5 ml/glass). The fractions D_1 (glass 30 – 36 incl.) and D_2 (glass 40 – 50 incl.) were after urea removal and further homogeneity tests (see text) sequenced. The bridge chromophore at about 250 nm was visible in the fractions D_1 and D_2 (as well as in E_{1a} and E_{1b}).

Determination of the N*-terminal sequence of the* ^{14}*C-labelled peptides.* The peptides D_1, D_2, E_{1a}, and E_{1b}, which represent *ca.* 90 % of the radioactivity, were sufficiently pure for sequence analysis by the criteria mentioned above (see section on methods). In each cycle of the Edman procedure, two DNS-amino acids appeared which shows the presence of two different, but non-separable, chains. On completion of each cycle, the phenyl-thiohydantoins were collected and counted on the low level apparatus. In Table 6, the ratio of the disintegration rate to the standard deviation of the counting technique is related to the amino acids found in each cycle. These results are compared with the known amino acid sequence of CT. The relevant segments of the B- and C-chains are shown in Fig. 9. In this way, the sites of modification could

Table 6. Identification of amino acid side chain carrying ^{14}C-labelled covalent bridge which connects two δ-CT monomers. The exclusive location of ^{14}C in His-57 and Met-192 was proven by isolation of ^{14}C-PTH which sequentially was released from the four peptides. The residual peptide did not contain significant amounts of the tracer in any of the four sequencing experiments The loss of ^{14}C occurred primarily during the preparation of the reagent and the modification of CT. The purity of the peptides was tested by examination of the elution profile, by electrophoresis and by observation of the number of amino acids which appeared after each cycle of the sequencing procedure. A trace of Tyr was ascribed to the adsorption of a small amount of this amino acid. Tryptic release of Tyr-146 (C-terminal after slight autolysis of δ-CT) was anticipated since the next neighbour is Arg. The peptides sequenced account for 90 % of the labelled protein.

Peptide[a]	Amino acid No.[b]	cpm./s_d[c]	Significance [d]	DNS-Edman [e]
D_1	1	5.5	$1:1.7\times10^6$	His*, Met*
	2	0.9	1:1.7	CM-Cys, Gly
	3	0.6	—	Gly, Asp
	4	0.2	—	Val, Ser
	5	0.2	—	Thr, Gly
	6	0.8	1:1.4	Thr, Gly
	Residual	1.5	1:6.5	—
D_2	1	3.2	1:727	His*, Met*
	2	0.2	—	CM-Cys, Gly
	3	0.2	—	Gly, Asp
	4	0.2	—	Val, Ser
	5	0.2	—	Thr, Gly
	6	0.8	—	Thr, Gly
	Residual	0.4	1:1.4	—
E_{1a}	1	1.4	1:5.2	Ala, Met*
	2	1.7	1:10.2	His*, Gly
	3	0.6	—	CM-Cys, Asp
	4	0.8	1:1.4	Gly, Ser
	5	0.1	—	Val, Gly
	6	0.1	—	Thr, Gly
	Residual	0.6	—	—
E_{1b}	1	4.4	$1:8\times10^5$	His,* Cys
	2	2.0	1:21	CM-Cys, Met*
	3	—	—	Gly, Gly
	4	—	—	Val, Asp
	5	—	—	Thr, Ser
	6	—	—	Thr, Gly
	Residual	—	—	—

[a] The peptides are electrophoretically homogeneous at pH 6.5 (30 min). [b] From NH-terminal (1). [c] Measured on isolated PTH, s_d = Standard deviation of mean. [d] Significance of decay rate data in previous column, *i.e.* of presence of ^{14}C in the aminoacid. [e] Subtractive Dansyl-Edman method.[26] [f] Acid hydrolysis apparently cleaves the cross-bridge without affecting the mobility of the amino acids. The amino acids which are expected to be labelled are marked with an asterisk. The chromatographic mobilities of the labelled amino acids indicate that ε-DNS-His and ε-DNS-Met were obtained and that the presumed cross-bridge either was cleaved without affecting the thioether function or the imidazole ring or failed to change the mobilities significantly.

Covalent sidechain linkage between 2 molecules of δ-chymotrypsin.

```
      57   58   59    60   61   62
... His . Cys . Gly . Val . Thr . Thr ... B-chain
          |
          CM
          |
... Met . Gly . Asp . Ser . Gly . Gly ... C'-chain
    192' 193' 194' 195' 196' 197'
```

Fig. 9. Summary of the amino acid sequence results and the ^{14}C-PTH-amino acid data showing the unique sequences which contain the bridge(s). The prime refers to the neighbouring molecule.

uniquely be assigned to His-57 and Met-192 (see Fig. 10). The amino acid composition analyses of the peptides are consistent with this finding.

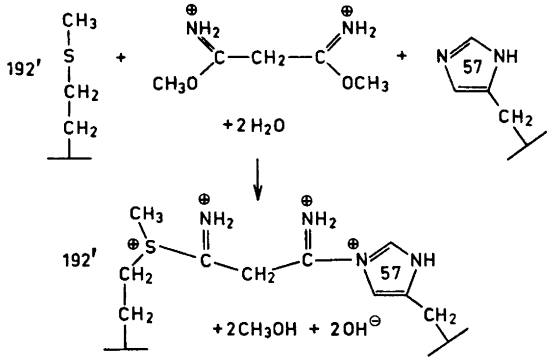


Fig. 10. Reaction scheme favoured by the accumulated evidence. It requires the presence of two analogous bridges which connect the two molecules of δ-CT.

Optical rotatory dispersion of MCT and CT. The difference ORD-curve between the modified enzyme and CT displayed small anomalies in the region of the aromatic side chains (260 – 270 nm). This range also corresponds to the absorption bands of the new chromophore in MCT and may be due to small Cotton effects. These, however, were so small that it was difficult to extract further information from them. The corresponding difference spectrum in the UV range between MCT and CT displayed, in addition to the chromophore already mentioned, also a band at 290 nm resembling the one reported by Wootton and Hess [39] as characteristic of the acyl enzyme. The origin of the effect was identified as a single Trp residue.

Stabilizing effect of the modification of the enzyme on the active conformation. MCT and α-CT were reduced and alkylated as described above. The A-chain and the by-products of the reaction were removed by dialysis and the kinetic parameters for the catalyzed hydrolysis of a specific substrate, *N*-acetyl-L-Tyr, were then determined. The results are listed in Table 7. Although the specificity coefficient, k_{cat}/K_m^{app}, is much diminished after the cleavage

of the disulfide linkages, a much greater fraction of the active sites remain intact in MCT than in α-CT.

Table 7. Stabilizing effect of cross-bridge in MCT. The disulfide bridges in δ-CT and MCT were reduced by Cleland's reagent and alkylated (see text). The three experiments with the reduced and alkylated MCT were carried out at different times after the dialysis. The experimental error listed is the standard deviation. All parameters were evaluated by linear regression using about 8 observations at different substrate concentrations. The UV-spectrum of the preparation used in exp. 2 showed that the protein still was native ($A_{280}/A_{260}=1.76$).

Enzyme	$\frac{k_{cat}}{K_m^{app}}$ sec^{-1} mM^{-1}	k_{cat} sec^{-1}	K_m^{app} mM	Relative efficiency
α-CT[a]	215	236	1.1 ± 0.3	1
δ-CT[b]	211	201	0.95	1.0
MCT[a]	740	519	0.7 ± 0.2	3.4
δ-CT, reduced and alkylated[a]	0.2	0.22 ± 0.02	1.0 ± 0.4	1
MCT, reduced and alkylated exp. 1[a]	0.78	0.86	1.1	4
MCT, reduced and alkylated exp. 2[c]	97 ± 7	448 ± 18	4.6 ± 0.3	490 ± 38
MCT, reduced and alkylated exp. 3[a]	34	17	0.5	68

[a] 25°C, pH 8.0, *N*-Ac-L-tyr-OEt, 0.1 M KCl. [b] pH 8.5, 0.45 M KCl. [c] pH 8.0, 0.04 M Tris, 20°C.

DISCUSSION

The evidence for the structure of the product of the reaction of dimethyl malondiimidate with *N*-acetyl-L-Met is the following:

1. The only function in *N*-Ac-L-Met which can react with an imidate is the thioether since the masking of the amino group was effective. This was checked by IR-measurement and by dansylation.
2. The kinetics supports the proposed molecularity.
3. The rate constant for formation of the derivative has the magnitude which could be expected from a reaction of the type suggested.
4. A small amount of homoserine was detected in the hydrolysate of MCT but not of CT. This indicates that Met has been attacked.
5. The spectral changes following the addition of the imidate to the *N*-Ac-Met or the enzyme could not be due to hydrolysis of dimethyl malondiimidate since the small spectral contribution of the latter at the wavelength of observation was subtracted.
6. Sulfonium compounds are known to exhibit multiple bands in the range where the Met derivative and MCT display such bands.[40]
7. Construction of the molecular model of the suggested Met derivative shows that the formation of this compound is feasible and plausible.
8. The alternative hypothesis: The existence of a strong ionic linkage between sulfonium and the imidate is excluded by the observation of the persistence of the characteristic band in MCT after thorough purification of the latter in the presence of a high concentration of salt and urea.

The conclusion must be that both MCT and the model compound contain the thioimidate group.

In the case of the His derivative, the evidence is:

1. The only group in *N*-Ac-L-His which can react with an imidate is the imidazole ring, since the masking of the amino group was effective. This was checked by IR- and NMR-measurement and by dansylation.

2. The expected molecular weight and decomposition pattern were found for the His derivative by mass spectrometry [33,34] (see Table 3). Fragments larger than the reactants were found.

3. The changes in spectrum and p*K*-values (see Figs. 3 and 8) show that a reaction has occurred and that the ring is involved. The spectrum resembles that of an amidine.

4. The elemental analysis and molecular weight determination from the colligative properties exclude other possible structures.

5. ^{14}C-labelled His was found in a partial hydrolysate of the modified enzyme.

The reported failure of previous attempts of modifying imidazole with an imidate may be explained by the difference in pH of the solution and in the reactivity of the reagents.[12] Amidination reactions are known to possess sharp pH-optima.[43] Since imidates require a nucleophile, only the nitrogen atoms of the ring need to be considered as reaction sites. The NMR-data are compatible with thus suggestion. The shielding effect on the imidazole ring of the His derivative (see Table 1) may be due to its participation in the mesomerism of the amidine. The diamagnetic shift of the protons on the α- and β-carbon atoms may be due to either an inductive effect or to the high electric field at the bridge. The latter is the more likely explanation since the α-C, which displays the larger deshielding effect can approach the bridge closer than the β-C can. The NMR-data are compatible with the hypothesis of the formation of the bridge and no plausible alternative seems apparent.

The incorporation ratio (see Table 5) shows that each CT-monomer carries one molecule of the modifying reagent. The sites of modification were identified as Met-192 and His-57 by amino acid sequence analysis of peptides which had been carefully purified and satisfied the homogeneity criteria. Since the disulfides were exhaustively reduced and carboxymethylated prior to the limited proteolysis and the fractionation and since the two modified amino acids are located in two different peptide chains (B and C), their presence in the same molecule shows that the two chains have become connected by a covalent bridge. No other amino acids than the two mentioned carried the ^{14}C-label in the four peptides which were sequenced and which represent 90 % of the label in the protein. Since all ether amino acids remained intact, the covalent bridge must connect Met-192 and His-57 directly. No homologous bridges (His-His or Met-Met) were found in the four peptides which were sequenced and an examination of the total model of the enzyme revealed that the formation of two such bridges as required by the measured incorporation ratio, was highly unlikely.[36]

An intramolecular His-57-Met-192 bridge is feasible in δ-CT but it is likely to impede the binding of specific substrates. Since both *N*-acetyl-L-Trp and *N*-acetyl-L-Tyr were good substrates of MCT and since the molecular weight

determinations indicated the existence of the covalent dimer, this structure was favoured. An examination of the model shows that the formation of a dimer stabilized by two covalent malono-bridges is feasible and plausible. The suggested bond types in the bridge are shown in Fig. 10.

In the dimer model, there is free access for a specific substrate molecule to either of the two active sites and they can be charged simultaneously and act in mutual independence. This is in keeping with the finding of 1.0 active site per molecule of monomer (see Table 5) by the cinnamoyl-imidazole titration.

The reactivity of *N*-acetyl-L-His and of *N*-acetyl-L-Met toward dimethyl malondiimidate shows that the suggested type of modification of CT should occur unless there are special difficulties, such as local steric hindrance or unfavourable electronic conditions. The CT-model does not exhibit any obvious features of that nature. On the contrary, both side-chains are located on the surface and in a position suitable for a bridge formation. Such a bridge would be expected to stabilize the active site, and it was actually possible to cleave the disulfide bridges and carboxymethylate them without loss of catalytic activity toward ATEE under standard assay conditions (see Table 7).

The direct observation of the characteristic thioimidate band in the spectrum of MCT at alkaline pH supports the involvement of Met in the modification reaction. The failure of the usual reaction with dimethyl malondiimidate after selective destruction of the Met-192 side chain by oxidation with performic acid suggests that this residue is involved. Amino acid analysis of MCT shows that one of the two methionines remain intact after the diimidate reaction and the location of the tracer identifies Met-181 as the noninvolved.

Direct evidence of the involvement of His-57-imidazole ring, other than the sequencing and amino acid composition analysis, is more difficult to obtain. A comparison between the p*K*-values of His in the model compound, in CT and in MCT is not easy because the correct assignment of these values to specific groups in the absence of high resolution NMR-data is uncertain. However, since the imidazole ring by amidination should become more basic and since base catalysis undoubtedly is an important part of the mechanism of chymotryptic catalysis,[41] the existence of a His-57-ring amidine in MCT offers a reasonable, partial explanation of the observed enhancement of the catalytic efficiency. The position of the side chain of His-57 in the molecule, which is influenced by hydrogen bonds between Asp-102-carboxylate and N_1 and between Ser-195-OH and N_3, suggests that the latter, rather than N_1, forms the amidine. The ratios of the shielding effects are consistent with the hypothesis that also in the model compound it is N_3, and not N_1 which reacts.

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