*APPROACHES TO THE SELECTIVE CHEMICAL LABELING OF THE ACTIVE SITE OF CARBOXYPEPTIDASE A**

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The identification of pancreatic carboxypeptidase A as a zinc metalloenzyme has opened new experimental approaches to the elucidation of the mechanism of action of this exopeptidase and to the role of the metal in the specificity and catalytic function of the enzyme.[1-3] Since zinc, or one of certain other metals in the first transition series, is essential for enzymatic catalysis,[4, 5] the site of binding of the metal to the protein constitutes an integral part of the active site.

Recent work by Vallee *et al.*[6, 7] has demonstrated that in bovine pancreatic carboxypeptidase A, prepared from an acetone powder of the glands, zinc is bound to the apoenzyme through a sulfhydryl group, obviously contributed by a cysteinyl residue. The evidence for the involvement of the thiol group is based on the differential reactivity of the native enzyme and the zinc-free enzyme towards silver, p-mercuribenzoate, and ferricyanide. With each of these reagents, one thiol group is detected in the metal-free enzyme, but not in the native protein. We have confirmed these findings by silver titrations. The existence of the metal mercaptide binding site is supported further by the order and magnitude of the stability constants of a series of metallocarboxypeptidases, complexometric titrations, and the spectrum of cobalt carboxypeptidase.[5-7] Recent work has provided evidence that the nitrogen of the α-amino group of asparagine at the N-terminus of the protein constitutes the second ligand of the metal besides the sulfur.[8]

The well-known reactivity of cysteine or cysteinyl peptides with alkylating agents has resulted in their wide application for the detection of cysteinyl residues in proteins. Yet neither iodoacetate, iodoacetamide, N-ethylmaleimide, nor DDPM[9]‡ reacts readily with the thiol group of the metal-free apoenzyme even after treatment with denaturing agents such as urea, sodium dodecyl sulfate, or BRIJ-35. However, if carboxypeptidase was first heat-denatured to render it susceptible to proteolytic degradation, and then digested with chymotrypsin, the thiol group could be readily alkylated, provided that the metal was first removed from the digest by the addition of o-phenanthroline (Table 1). Thus, only under conditions of extensive disruption of the structure of the apoenzyme can the thiol group of the active

TABLE 1

Reaction of Iodoacetamide with Carboxypeptidase A

Enzyme	Treatment prior to alkylation*	CM-Cys (residues/molecule)
Native	.....	0
Metal-free†	.....	0
Heat-denatured	Chymotryptic digestion‡	0
Heat-denatured	Chymotryptic digestion + o-phenanthroline‡	0.93
Heat-denatured	Chymotryptic digestion + β-mercaptoethanol‡	1.93

* 2–5 fold excess of iodoacetamide at pH 8.0 in 1.0 *M* NaCl.
† One residue of cysteine by titration with silver or p-mercuribenzoate.[7]
‡ Digested under nitrogen with molar ratio of chymotrypsin/carboxypeptidase A of 1/100 at pH 8.0 for 60 hr at 37°C.

site of carboxypeptidase be made to react with alkylating agents in a manner characteristic of sulfhydryl groups of model compounds of simpler structure.

Although an alkyl label could be introduced onto the zinc-binding thiol in this manner, the advantage was lost of correlating the extent of introduction of the label with the disappearance of enzymatic activity. To permit this correlation, an approach was sought whereby the label could be introduced into the intact protein. In these and all of the following experiments, bovine pancreatic carboxypeptidase A, isolated from an acetone powder prepared from freshly collected glands, was used.[10]

The amino acid composition of carboxypeptidase A was determined by Smith and Stockell,[11] by Hill and Schmidt,[12] and in our laboratory.[13, 14] Of the 307 amino acid residues of which the enzyme is composed,[14] only five contain sulfur; three occur in the form of methionine, and two in a form which upon oxidation yields cysteic acid (Table 2). When the native enzyme was treated with the reducing agent, β-mercaptoethanol, and subsequently coupled with alkylating agents, such as iodoacetate, iodoacetamide, or DDPM, two equivalents of S-alkylcysteine were obtained after acid hydrolysis (Table 2). The same result was ob-

TABLE 2

SULFUR DISTRIBUTION PER MOLECULE OF CARBOXYPEPTIDASE A

Method	Derivatives of half cysteine residues*	Methionine residues*	Total sulfur atoms
Schoniger ignition[15]	..	..	5
Performic oxidation[16]	2.0	3.0	..
β-mercaptoethanol	..	..	..
+ iodoacetate†	1.9	2.9	..
+ iodoacetamide†	2.0	2.6	..
+ DPPM†	2.0‡	3.0	..
Acid hydrolysis	0–2.0	3.0	..

* Amino acid analyses performed on a Spinco Analyzer.

† Protein treated with 0.06 *M* mercaptoethanol at 0°C in 1.0 *M* NaCl, 4.0 *M* urea for 1 hr at pH 8.0, then coupled at 0°C with alkylating agent at pH 8.0 in 2 to 5 fold excess over thiol. In the case of DDPM, the alkylation was done at pH 4.6 for 2 hr at 37°C.

‡ Using an approximate correction factor for losses on 72 hr hydrolysis in 6 *N* HCl at 105°C.

tained when a chymotryptic digest of heat-denatured enzyme was used instead of the native enzyme (Table 1). Thus, β-mercaptoethanol renders the thiol of the active site available for alkylation and converts the second sulfur to a form which is analytically characterized as a cysteine residue. Treatment of carboxypeptidase with this reducing agent under the conditions employed is irreversible in that enzymatic activity cannot be restored by removal of the reagent and addition of the metal to the protein. Presumably, β-mercaptoethanol, besides competing with the nitrogen-zinc-sulfur ligand and exposing the cysteine, modifies the protein in such a fashion as to make this thiol available for subsequent alkylation. While one is tempted to speculate on the nature of the second sulfur in the native protein that gives rise to cysteine on reduction with β-mercaptoethanol, the problem, as regards our objective to label specifically the thiol group of the active site, resolves itself operationally into introducing one more thiol group into the protein molecule by treating the enzyme with a reducing agent, and subsequently differentiating this newly formed artifactitious thiol from the one that is bound to zinc in the native protein. To this end, advantage was taken of the enzymatic specificity of carboxypeptidase A by exposing it to a competitive inhibitor which protects the configura-

tion of the active site by preventing the effect of the reducing agent on the ligands and yet permits the reduction of the second sulfur to a cysteinyl residue.

Thus when carboxypeptidase A is treated with a competitive inhibitor, such as β-phenylpropionate,[17] and then reduced with β-mercaptoethanol and treated with iodoacetamide, the enzyme remains fully active and substantial alkylation of the newly formed thiol occurs. When the same reaction is carried out in the absence of the competitive inhibitor, enzymatic activity is rapidly lost (Fig. 1), and two

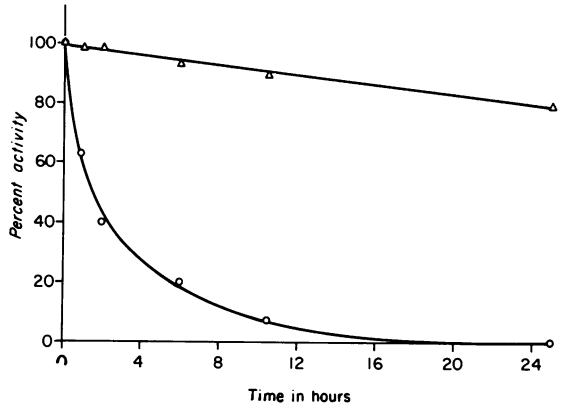

FIG. 1.—Protection of carboxypeptidase A by β-phenylpropionate against inactivation by β-mercaptoethanol. Reduction was carried out at pH 8 at 0° in the presence (Δ) and absence (○) of 0.1 *M* β-phenylpropionate. The reaction mixtures contained 0.33 *M* β-mercaptoethanol, 1 *M* NaCl, and 0.1 *M* Tris-HCl buffer. Carboxypeptidase, 2 mg/ml. Relative activities were measured using hippurylphenylacetate as substrate.

equivalents of carboxymethylcysteine are formed on alkylation of the reduced protein (Table 2). This protection of the enzyme by β-phenylpropionate from inactivation by β-mercaptoethanol is dependent on the inhibitor concentration and occurs in a range where, according to kinetic evidence,[17] the inhibitor is tightly bound (10^{-2} to 10^{-1} *M*). The selectivity of the reaction is influenced by pH and by the presence of urea in the reaction mixture. While 4 *M* urea was present in most of the earlier experiments, the reaction can be made to occur even in the absence of urea by using five times higher concentrations of β-mercaptoethanol. Pertinent experimental data are summarized in Table 3. β-Phenylpropionate has no pro-

TABLE 3

ALKYLATION OF THIOLS OF CARBOXYPEPTIDASE A

Carboxy-peptidase A	Urea	β-phenyl-propionate	Reducing agent*	pH	Enzyme inactivation† %	CM-Cys (residues/ molecule)
+	+	−	ME 0.06 *M*	8.0	100	2.00
+	+	+	ME 0.06 *M*	8.0	5	0.40
+	−	+	ME 0 33 *M*	7.2	0	0.56
+	−	+	ME 0.33 *M*	8.0	2	0.44
+	−	−	$NaBH_4$	9.0	26	1.10
+	−	−	$NaBH_4$‡	9.0	48	1.44
+	−	+	$NaBH_4$	9.0	0	0.95

* The protein was treated with mercaptoethanol in the presence of 0.1 *M* β-phenylpropionate and then alkylated with iodoacetamide as in Table 2. Reduction with 1 per cent sodium borohydride was done at 0° for 22 hr in 2 *M* NaCl, 0.2 *M* Tris-Cl, 0.2 *M* β-phenylpropionate on solutions containing 2 mg carboxypeptidase per ml. The protein reduced with borohydride was alkylated for 2 hr at 0° by adding 5.3 mg iodoacetamide per ml.

† Activities were measured with hippurylphenylacetate as the substrate.

‡ Protein incubated for 16 hr with 1.9 per cent sodium borohydride.

tective effect on the metal-free apoenzyme and hence two cysteines become available for alkylation on treatment with β-mercaptoethanol. This is in accord with earlier observations[18] that an analog of β-phenylpropionate, phenylacetate, is not bound by the metal-free apoenzyme, and with the recent demonstrations by Coleman and Vallee[19] that β-phenylpropionate is not bound either by the apoenzyme.

When in the preceding experiments, β-mercaptoethanol is replaced by sodium borohydride, the formation and subsequent alkylation of the artifactitious thiol becomes quantitative and fully selective, as evidenced by the fact that the monoalkyl protein retains full enzymatic activity. Under more vigorous conditions of treatment with sodium borohydride, in the absence of β-phenylpropionate, enzymatic activity is progressively lost and this loss can be correlated with additional yields of carboxymethylcysteine over and above the one equivalent contributed by the newly formed thiol (bottom of Table 3).

On the basis of the experiments just described, it has been possible to label selectively the thiol of the active site, and to isolate the labeled peptides. Two reagents were found to be most suitable to this end. One of these is iodoacetate, containing 1-C^{14}, and the other reagent is Witter and Tuppy's yellow derivative of N-ethylmaleimide, DDPM.[9] The yellow peptides could be separated by selective adsorption on talc whereas the radioactive peptides were separated by chromatography on Dowex 50 X 2. High voltage electrophoresis and paper chromatography were used for further purification of the peptides thus obtained. The amino acid composition of the labeled peptides was determined after isolating them from Nagarse digests of the labeled protein either as colored DDPS-derivatives or as C^{14}-carboxymethylcysteine peptides.

Selective labeling of the thiol at the active site with DDPM was achieved as follows: The protein was first reduced with sodium borohydride in the presence of β-phenylpropionate, and the newly formed thiol blocked with iodoacetamide. The monoalkyl-carboxypeptidase so obtained, which was fully active, was then treated with β-mercaptoethanol and subsequently alkylated with DDPM, which could couple only with the thiol group that bound zinc in the native protein. This protein was digested with Nagarse, and several yellow peptides were isolated from the digest. The major peptide had the composition (Cys,Ser_2) as indicated at the top of Table 4. Thus, (Cys,Ser_2) represents the peptide containing the sulfur ligand of the active site.

In contrast to this selective approach of labeling the active site thiol, the same alkylating reagent was used to react with both the intrinsic thiol and the artifactitious

TABLE 4

LABELED CYSTEINYL PEPTIDES FROM NAGARSE DIGESTS OF CARBOXYPEPTIDASE A

Carboxypeptidase A	Isolated peptides	
Active center thiol alkylated with DDPM after the second sulfur was blocked with iodoacetamide*	(DDPS-Cys,Ser_2) (DDPS-Cys,Ser_2,Glu)	Trace of (DDPS-Cys,Gly,Val)
Both thiols alkylated†	(DDPS-Cys,Ser_2) (DDPS-Cys,Ser_2,Glu) (C^{14}-CM-Cys,Ser_3,Glu,Asp,Gly) (C^{14}-CM-Cys,Ser_4,Gly,Asp,Gly_2)	(DDPS-Cys,Val,Gly) (DDPS-Cys,Val,Gly,Asp) (C^{14}-CM-Cys,Val_2,Gly_2,Asp,Glu)

* The fully active, monoalkyl-carboxypeptidase A (Table 3, bottom line) was treated with 0.06 *M* mercaptoethanol in 4 *M* urea and alkylated with DPPM.
† Alkylated as in Table 2.

thiol that resulted on treatment of the protein with β-mercaptoethanol in the absence of β-phenylpropionate. Iodoacetate-1-C^{14} or DDPM was employed in these studies. The amino acid compositions of the labeled peptides isolated from Nagarse digests of such labeled proteins are summarized at the bottom of Table 4. As is to be expected, two families of peptides are found—one similar to the peptide at the active site (top line of Table 4), the other obviously representing the region of the second sulfur which became *reduced* and alkylated in the intact protein. The amino acid sequences in the various cysteinyl peptides and their linear relation to each other in the parent molecule are now under investigation.

Discussion.—The present investigation reveals an illuminating aspect of the thiol of the active site in carboxypeptidase A. This sulfhydryl group reacts readily with silver, p-mercuribenzoate, and ferricyanide; yet it does not react with alkylating agents which readily couple with sulfhydryl groups in some proteins and in model compounds of simpler structure.[20, 21] It is only after extensive irreversible treatment of the protein (e.g., β-mercaptoethanol or chymotryptic hydrolysis) that the thiol becomes available for alkylation. This lack of reactivity may well relate to details of the tertiary configuration of the apoenzyme and to interactions of the sulfhydryl groups with neighboring groups such as have been encountered with other proteins.[20, 21] Thus the behavior of this group constitutes yet another example of the limitation of group specific and selective reagents in that failure to alkylate a suspected thiol group does not rule out its presence. This lack of alkylation may be an inherent property of certain protein sulfhydryl groups which is inadequately simulated by model compounds of simpler structure. Whatever the explanation for the lack of reactivity of the thiol group of apocarboxypeptidase to alkylating agents, the phenomenon itself has required that through the addition of a reducing agent the thiol of the active side be made reactive towards alkylating agents; however, at the same time, the second sulfur is *reduced* and made available for alkylation. The action of the reducing agent is obviously different in each case since the thiol of the active site is an intrinsic attribute of the enzyme,[7] whereas the other thiol is an artifact of the analysis and is created only as a result of reductive procedures employed in this work. After its formation, the artifactitious thiol can be differentiated from the pre-existing one by the fact that, as to be expected, it is neither involved in enzymatic function nor affected by inhibitors which combine specifically with the active center of the enzyme. The chemical nature of the second sulfur in the native enzyme remains still in doubt. All that can be said at the moment is that it is definitely not a cysteine side chain and probably not a thiolester since treatment with hydroxylamine under conditions usually employed for the hydrolysis of thiolesters[22] does not yield a thiol reactive with iodoacetate, even in the presence of urea.

The hazards of identifying the amino acid residues in proteins by analysis of protein hydrolysates are clearly illustrated by the nature and behavior of the sulfur atoms in carboxypeptidase. While three of the sulfurs are evidently derived from methionine residues, analyses of the hydrolysates of the reduced and alkylated enzyme, or of the oxidized enzyme, invariably point to the existence of two half-cysteines in the intact proteins which cannot be differentiated from each other by these conventional procedures. Conventionally, finding of the second cysteic acid residue in the hydrolysate would lead to the reasonable conclusion that it is derived

from a cysteine in the primary structure. The contradictory data here obtained by means of site specific reagents on one hand and by analysis of the oxidized protein on the other led to the investigations which revealed that in this instance, at least, analysis of the protein hydrolysate fails to represent accurately the chemical nature of the sulfur containing amino acid residues. The data suggest that a hitherto unrecognized group or residue seems to account for what is only an apparent discrepancy between the results of amino acid analysis and of site specific reagents.

The inadequacy of compounds of simpler structure to serve as models of specific functional groupings of enzymes is not restricted to carboxypeptidase alone. Thus, while cysteine or cysteinyl peptides can be readily alkylated, the thiol of the metal-free carboxypeptidase cannot. In contrast, the single site which makes certain esterases such as trypsin or chymotrypsin so reactive towards acylating or phosphorylating reagents is commonly identified with the hydroxyl group of a serine side chain.[23, 24] Yet neither serine or seryl peptides, nor the score of other seryl side chains in the same enzymes, react similarly with these reagents. In an analogous situation, the histidyl group at the active site of ribonuclease has only been recognized on the basis of an unusual pH dependence and reactivity to carboxymethylation.[25] It is only through analytical approaches which take advantage of specific interactions that the functional group—the thiol which binds the metal in carboxypeptidase, the hydroxyl which becomes phosphorylated or acylated in the esterases, and the imidazolyl group that can be carboxymethylated in ribonuclease —can be recognized and differentiated from other groupings which, although seemingly identical after destruction of the protein, actually are different.

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‡ The following abbreviations are here used: ME = β-mercaptoethanol, DDPM = N-(4-dimethylamino-3,5-dinitrophenyl) maleimide, DDPS-Cys = the adduct of DDPM and cysteine, CM-Cys = S-carboxymethyl cysteine.

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A PHSYICAL BASIS FOR DEGENERACY IN THE AMINO ACID CODE

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If two or more nucleotide sequences specify the same amino acid, the code is said to be degenerate for that amino acid. Evidence that such degeneracy exists in *E. coli* comes from experiments on the stimulation of ribosomal incorporation of amino acids by synthetic polyribonucleotides. For instance, Martin *et al.*[1] and Speyer *et al.*[2] found that either poly UC or poly UG stimulates incorporation of leucine, so that at least two different combinations code for the same amino acid. Since the specificity of coding seems to reside in sRNA molecules functioning as adaptors,[3, 4] such degeneracy might be due to the existence of two sRNA acceptors for leucine, each having different coding properties. Two peaks of leucine-acceptor activity were demonstrated in yeast sRNA by Doctor *et al.*[5] using countercurrent distribution. Berg and Lagerkvist[6] have shown that *E. coli* sRNA also contains two leucine acceptors with different acceptor-terminal nucleotide sequences. In this paper, two leucine acceptors in *E. coli*, separated by countercurrent distribution, are shown to have different coding properties. One responds preferentially to poly UC, the other to poly UG.

Materials and Methods.—Separation of two leucine acceptors: sRNA from *E. coli*, strain KB, was prepared by phenol extraction and passage over DEAE cellulose as described by Holley *et al.*,[7] then stripped of amino acids, dialyzed, and lyophilized, as described by von Ehrenstein and Lipmann.[8] Countercurrent distribution was done exactly as described by Apgar *et al.*[9] 200 mg of *E. coli* sRNA was added to the first 5 tubes of a 200-tube apparatus, and the final fractions were combined into 40 sets of 5 tubes each, dialyzed, recovered by evaporation, and each dissolved in 2 ml of deionized water. A 100,000 × *g* supernatant of an *E. coli* extract was used as the source of amino acyl RNA synthetase activity. For assays of acceptor activity of the sRNA fractions, the reaction mixture contained in a volume of 0.5 ml: Tris-HCl, pH 7.2, 50 μM; $MgCl_2$, 5 μM; KCl, 5 μM; adenosine triphosphate, Na salt (ATP), 0.5 μM; 0.2 μc of C^{14}-amino acid, either DL-