AMINO ACID SEQUENCE AROUND THE REACTIVE CYSTEINE RESIDUES IN THIOLASE

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1. Introduction

Thiolase (EC 2.3.1.9) plays an important part in the biological oxidation of fatty acids (for review see ref. [1]), and the observation that the enzyme was inactivated by reagents known to react with thiol groups led Lynen [2] to propose that thiol groups were involved in its catalytic activity. The pure enzyme from pig heart [3] has been shown to contain *at least* three reactive cysteine residues per mole (170 000 × *g*) [4] of enzyme protein. These reactive cysteines serve as acyl acceptor groups during the thiolytic cleavage of acetoacetyl-CoA as well as in the acyl transfer reaction. Thus incubation with acetyl-CoA leads to the incorporation of acetyl groups into the enzyme protein [3]. In the presence of the substrates acetoacetyl-CoA and acetyl-CoA inhibition of thiolase and acetyl transferase activities by iodoacetamide does not occur. This suggests that the inhibitor reacts with cysteine residues which are essential for enzymatic activity.

The present communication describes the isolation and characterisation of a unique heptapeptide, containing [1-^{14}C] carbamoylmethylcysteine, and [1-^{14}C] acetylcysteine, respectively, derived from tryptic digests: a) of enzyme which had been inactivated with [1-^{14}C] iodoacetamide; and b) of the [1-^{14}C] acetylenzyme formed by reaction with [1-^{14}C] acetyl-CoA.

Non-standard abbreviations
CMCys, *S*-carboxymethylcysteine
CAMCys, *S*-carbamoylmethylcysteine
Cya, Cysteic acid

2. Reaction with [1-^{14}C] iodoacetamide

Thiolase was prepared from pig heart muscle as previously described [3]. The enzyme (0.2 μM) was reacted with [1-^{14}C] iodoacetamide (0.65 μmole, containing 5.0 mc/mM) in 0.06 M K-phosphate buffer (8 ml) at pH 7.0 and 0°. After 20 min 53% of the original activity had been lost and the reaction was terminated at this stage by addition of excess mercaptoethanol. The mixture was dialysed at 4° against several changes of 0.01 M K-phosphate (pH 7.0), freeze-dried, and redissolved in 6M guanidine-HCl (4 ml, containing 0.4 m moles tris-HCl and 2 μmoles dithiothreitol) at pH 8.3. This solution was incubated at 37° for 2 hr; the fully reduced protein was then reacted with an excess (15 μmoles) of iodoacetic acid at 20° for 3 hr, dialysed against mM HCl and stored frozen at -10°.

A separate experiment was carried out under the same conditions, except that the concentration of [1-^{14}C] iodoacetamide was increased threefold, and the reaction was allowed to proceed for 30 min, that is until less than 1% of the enzyme activity remained. The two preparations of carbamoylmethyl-enzyme contained 2.3 and 4.1 g atoms [^{14}C], respectively, per mole (170 000 g) of protein.

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3. Reaction with [1-^{14}C] acetyl-CoA

Thiolase (0.1 μmole) dissolved in 0.04 M K-phosphate (4 ml, containing 5 μmoles dithiothreitol) at pH 7.0 was allowed to react with [1-^{14}C] acetyl-CoA (4 μmoles containing 5.0 mc/mM) at 0° for 10 min The reaction was terminated by adding cold acetone-HCl (100 ml containing 0.5 ml 12 N HCl). The precipitated protein was washed several times with cold acetone, air-dried, redissolved in 5 M guanidine-HCl (3 ml, adjusted to pH 3 with HCl), and finally dialysed at 4° against several changes of mM HCl. The acetyl-enzyme prepared in this way contained approximately 3.3 g atoms [^{14}C]/mole.

4. Isolation and sequence of a heptapeptide containing [1-^{14}C] carbamoylmethylcysteine

The carbamoylmethyl enzyme (2 mg/ml containing 2.3 g atoms [^{14}C]/mole) was digested with trypsin (2%) in a pH-stat at pH 8.5 and 20° for 14 hr. The resulting digest was submitted to gel filtration on Sephadex G-50 (142 X 2 cm column) in 0.1 M ammonium bicarbonate. Effluent fractions (5 ml) were analysed for [^{14}C] and their optical density at 225 mμ was also measured. As shown in fig. 1 (a) two radioactive fractions (T1 and T2 containing 44% and 52% respectively of the total counts applied to the column) were obtained. The same two radioactive fractions were obtained from the tryptic digest of the enzyme derivative (containing 4.1 g atoms [^{14}C]/mole) which had been completely inactivated with [1-^{14}C] iodoacetamide. In addition several minor radioactive fractions (which together contained no more than 10% of the total counts in the digest) were also found and were presumably due to reaction of other cysteine residues in the protein with [1-^{14}C] iodoacetamide (cf. ref. [3]). Fraction T2 gave rise to only one radioactive peptide (T2, m = + 0.35) by electrophoresis on paper at pH 6.5, and this was prepared in pure form by successive electrophoresis on paper at pH's of 6.5, 3.5 and 1.9 (cf. refs. [5,6]). It gave a single *N*-terminal group, valine, by the dansyl method [7,8]. Amino acid analysis (following hydrolysis with 6 N HCl at 105° for 24 hr) showed that it was a heptapeptide containing one residue each of lysine, [1-^{14}C] carboxymethylcysteine, serine, glycine, alanine, valine and methionine. All the [^{14}C] was present as carboxymethylcysteine and the amino acid sequence of the peptide was established to be Val-^{14}CAMCys-Ala-Ser-Gly-Met-Lys by the dansyl-Edman method [7].

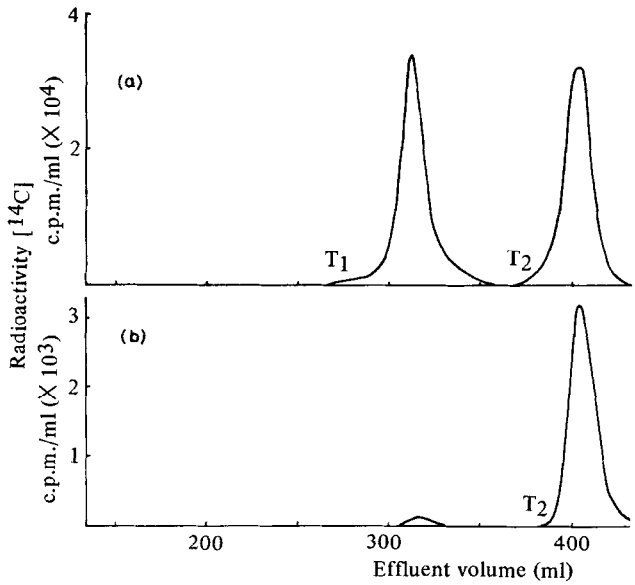

Fig. 1. Gel-filtration on Sephadex G-50 (142 X 2 cm) in 0.1 M ammonium bicarbonate of: (a) Tryptic digest of [1-^{14}C] carbamoylmethyl-thiolase. (b) Fraction T1 (1/10) redigested with trypsin and reapplied to the same column. For details see text.

Redigestion of fraction T1 with trypsin (2% for 6 hr at 37°), followed by gel filtration on the same column of Sephadex G-50, gave rise to a single radioactive peak in exactly the same position as fraction T2 (fig. 1 (b)). This material proved to be identical to the heptapeptide T2, showing that the presence of peak T1 in the initial digest was due to incomplete trypsin digestion of the carbamoylmethyl-enzyme. Inactivation of thiolase by iodoacetamide is thus shown to be due to its specific reaction with a unique cysteine residue in the primary structure of the enzyme protein.

5. Isolation and sequence of a peptide containing [1-^{14}C] acetylcysteine

The [1-^{14}C] acetyl derivative of thiolase (0.1 μmole, containing approximately 3.3 g atoms [^{14}C]/mole) in aqueous suspension (7 ml) at pH 6.7 was di-

gested with trypsin (1.5 mg) in a pH-stat at 20°. After 14 hr a considerable fraction of the digestion mixture remained insoluble; the digestion was therefore continued for an additional 6 hr at 37° and the still turbid solution was acidified to pH 3 with acetic acid. The precipitate was removed and the soluble fraction (which contained over 90% of the total radioactivity) was submitted to gel filtration on Sephadex G-50 (column dimensions 145 × 1.7 cm) in 5% acetic acid. The fraction (AcT) containing [^{14}C] was obtained as a single peak (effluent volume, 280-310 ml). This corresponds very closely to the effluent volume of peptide T2 from the iodoacetamide-inhibited enzyme eluted from the same Sephadex column in 5% acetic acid. Moreover electrophoresis on paper at pH 6.5 showed the main radioactive component in this fraction (ca. 90% of the total counts) to be a basic peptide with a mobility (m = +0.35 at pH 6.5) similar to that of the carbamoylmethyl-heptapeptide T2. Peptide AcT was subsequently purified by successive paper electrophoresis at pH's 6.5, 3.5 and 1.9. The pure [1-^{14}C] acetyl-peptide contained *N*-terminal valine. Oxidation with performic acid led to total loss of [^{14}C] (cf. ref. [5]) and amino acid analysis of the oxidised peptide showed it to be a heptapeptide of the same composition (except that it contained one residue of cysteic acid instead of carboxymethylcysteine) as peptide T2 from the iodoacetamide inhibited enzyme. Its relationship to T2 was confirmed when its sequence (determined by the dansyl-Edman method [7,8]) was established to be Val-Cya-Ala-Ser-Gly-Met-Lys.

These results show that the acetyl groups which are incorporated into thiolase during its reaction with acetyl-CoA are bound in thioester linkage to cysteine residues in the enzyme, and that these reactive cysteines occur in a unique sequence in the primary structure of the enzyme protein. Moreover the same cysteine residues have been found to react with iodoacetamide showing that the inhibitor functions by reacting irreversibly with the cysteine residues which are directly involved in the catalytic activity of the enzyme. In this respect thiolase resembles glyceraldehyde 3-phosphate dehydrogenase [5].

Enzyme preparations that were used in the present study were found to contain approximately 3 reactive cysteines per mole (170 000 × g). The active enzyme has however been shown to consist of *four* sub-units of similar size [4], and the present results (reinforced by the results of peptide mapping studies, Gehring and Harris, unpublished results) suggest very strongly that thiolase like glyceraldehyde 3-phosphate dehydrogenase [6] consists of four polypeptide chains of similar and probably identical amino acid sequence.

References

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