AN AMINO ACID SEQUENCE IN THE ACTIVE SITE OF LIPOAMIDE DEHYDROGENASE FROM THE 2-OXOGLUTARATE DEHYDROGENASE COMPLEX OF *E.COLI* (CROOKES STRAIN)

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1. Introduction

Lipoamide dehydrogenase (reduced nicotinamide adenine dinucleotide: lipoamide oxidoreductase EC 1.6.4.3.) is a component of the pyruvate and 2-oxoglutarate dehydrogenase multienzyme complexes of *E.coli*. When isolated from *E.coli* (Crookes strain) the lipoamide dehydrogenase components of these two complexes appear to be identical by several criteria [1] although a stringent comparison by amino acid sequence analysis has not yet been made. Lipoamide dehydrogenase from *E.coli* has been reported to contain a single disulphide bridge and three free cysteine residues in each subunit [2,3] and the disulphide bond is thought to be directly involved in the catalytic action of the enzyme, being alternately reduced and oxidized [4,5].

We have now found that the two cysteine residues forming the disulphide bridge in the active site of lipoamide dehydrogenase from the 2-oxoglutarate dehydrogenase multienzyme complex of *E.coli* (Crookes strain) can be specifically labelled with iodo [2-^{14}C] acetic acid. This paper describes the specific labelling technique and the amino acid sequence of a tryptic peptide of 17 residues containing the modified cysteine residues. The results provide convincing chemical evidence that a single disulphide bridge per subunit is involved in these reactions and indicate that the two subunits comprising the native enzyme have very similar if not identical primary structures.

Abbreviations:
SDS : sodium dodecyl sulphate;
Cmc : *S*-carboxymethylcysteine.

2. Materials and methods

2-Oxoglutarate dehydrogenase multienzyme complex was purified from *E.coli* (Crookes strain) and assayed by the method of Reed and Mukherjee [6]. Lipoamide dehydrogenase was assayed by the method of Reed and Willms [7]. Proteins were *S*-carboxymethylated in the presence of 6 M guanidine hydrochloride and 2 mM dithiothreitol as described by Gibbons and Perham [8] and polyacrylamide gel electrophoresis of proteins in the presence of SDS* was carried out, bands were fixed, and molecular weights estimated using the techniques previously described [9].

Tryptic and chymotryptic digestion, paper electrophoresis and chromatography, radioactivity measurements, amino acid analysis and dansyl-Edman degradation of peptides were carried out as previously described [10]. Peptide maps and radioautographs were prepared by the method of Harris and Perham [11] and amide assignments in peptides were made from the electrophoretic mobility (m) of the peptide at pH 6.5 [12], defining the mobility of aspartic acid as -1.00.

3. Results

SDS-gel electrophoresis of freshly prepared 2-oxoglutarate dehydrogenase complex gives three

bands with subunit molecular weights estimated as 95,000, 58,000 and 51,000, corresponding to 2-oxoglutarate decarboxylase, lipoamide dehydrogenase and lipoyl transsuccinylase, respectively [9]. After storage in frozen solution with occasional thawing, a preparation that had lost 2-oxoglutarate dehydrogenase activity still retained lipoamide dehydrogenase activity. SDS-gel electrophoresis showed that extensive degradation of the components of molecular weights 95,000 and 51,000 had occurred, producing a spectrum of lower molecular weight polypeptides and leaving intact only the lipoamide dehydrogenase component [9]. This enzyme was rescued as follows.

The solution was clarified by centrifugation and applied to a column of hydroxyapatite (2 cm × 10 cm) in 0.1 M potassium phosphate buffer, pH 7.0. After washing the column with the same buffer until the effluent was free of protein, the lipoamide dehydrogenase was eluted with 0.4 M potassium phosphate buffer, pH 7.0. (This technique was based on the unpublished experiments of Mr. R.A. Harrison). On SDS-gel electrophoresis, this material showed one band with molecular weight 58,000 and it was further identified as pure lipoamide dehydrogenase by virtue of its enzymic activity [7].

3.1. *Identification of the active site peptide*

A radioautograph of a tryptic peptide map of lipoamide dehydrogenase that had been reduced with dithiothreitol and *S*-carboxymethylated with iodo [2-^{14}C] acetic acid [8] showed three radioactive peptides with electrophoretic mobilities of −0.20, −0.48 and −0.56, together with some radioactive material at the origin of the map.

In a second experiment, lipoamide dehydrogenase was *S*-carboxymethylated with unlabelled iodoacetic acid without prior reduction by dithiothreitol, the crystalline guanidine hydrochloride being added to the protein solution after the iodoacetic acid. Following extensive dialysis against distilled water, the protein was recovered by freeze drying and then reduced and *S*-carboxymethylated with iodo [2-^{14}C] acetic acid in the presence of guanidine hydrochloride, under the usual conditions [8]. A radioautograph of a tryptic peptide map of this material showed only one radioactive peptide (m = −0.20), corresponding to the peptide of the same mobility in the previous experiment. It was therefore expected that this peptide contained two *S*-carboxymethyl-cysteine residues derived from the active site disulphide bridge. This expectation was supported by the results of amino acid analysis of the *S*-carboxymethylated proteins, which were consistent with three cysteine residues (per subunit M.W. of 58,000) having reacted in the first carboxymethylation and with two further cysteine residues (previously forming a disulphide bridge) reacting in the second alkylation after reduction.

3.2. *Purification and characterization of the active site peptide*

Lipoamide dehydrogenase (50 mg) was reduced and *S*-carboxymethylated with iodo [2-^{14}C] acetic acid, digested with trypsin and the peptides fractionated by gel filtration on a column of Sephadex G-50 superfine (120 cm × 2 cm) in 0.5% NH_4HCO_3. Radioactivity in the column effluent was determined by scintillation counting and the radioactive peptide with mobility (m) of −0.20 was located by submitting a sample from each fraction to paper electrophoresis at pH 6.5. The appropriate fractions were freeze-dried and the required peptide, designated KT4c, was purified by paper electrophoresis at pH 6.5 and pH 3.5. The amino acid composition is shown in table 1.

A chymotryptic digest of peptide KT4c was fractionated by paper electrophoresis at pH 6.5 and by paper chromatography and three peptides, KT4cC1, KT4cC2 and KT4cC3 were isolated. Their amino acid compositions are also shown in table 1.

The intact peptide KT4c and the three chymotryptic fragments were all characterized by means of the dansyl-Edman degradation and amide assignments were made from the electrophoretic mobilities of the peptides [12]. The order of the carboxyl-terminal two residues of peptide KT4cC2 is deduced assuming lysine to be the *C*-terminus of the intact tryptic peptide KT4c. The results enable a unique sequence for peptide KT4c to be established as shown in fig. 1, since peptides KT4cC1, KT4cC2 and KT4cC3 together account for the whole of peptide KT4c. Further, the lack of heterogeneity in the determined sequence and the results of the peptide mapping indicate that the two subunits comprising the native enzyme have very similar if not identical structures. The same conclusion has been reached elsewhere [13].

Table 1
Amino acid compositions and electrophoretic mobilities of peptide KT4c and the three chymotryptic peptides derived from it.

Amino acid	Peptides			
	KT4c	KT4cC1	KT4cC2	KT4cC3
Lys	0.99		1.00	
Cmc	1.36(1.96)	0.81(1.01)	0.41(0.94)	
Asp	2.00		0.89	0.98
Thr	0.94			1.00
Ser	1.01		1.02	
Pro	1.04		0.89	
Gly	2.87	2.00	1.02	
Val	1.88	0.98	0.87	
Ile	0.97		0.82	
Leu	1.92	1.03		1.07
Tyr	0.93			0.98
Mobility at pH 6.5 (m)	−0.20	−0.45	0.00	0.00

The peptides were purified as described in the text. Compositions are given as moles/mole peptide. The values for Cmc given in brackets were determined by scintillation counting.

4. Discussion

The complete sequence of peptide KT4c is shown in fig.1. The clear cut results of the labelling experiments with iodo [2-^{14}C] acetic acid indicate that thiol-disulphide interchange has not taken place and that a unique disulphide bridge is involved in these reactions. Part of this sequence has also been determined independently for lipoamide dehydrogenase from *E.coli* B by other workers using different techniques [13], details of which appeared after the completion of the experiments described in this paper. The two sets of results agree perfectly for the region in common, lending further conviction to the belief that a unique disulphide bridge is involved in the catalytic mechanism of the enzyme. Since the present work was undertaken with lipoamide dehydrogenase specifically from the 2-oxoglutarate dehydrogenase complex of *E.coli* (Crookes strain) whereas the other experiments [13] were carried out with lipoamide dehydrogenase isolated direct from *E.coli* B, the fact that the active site sequences obtained are identical over the 11 residues in common suggests that the lipoamide dehydrogenases from pyruvate and 2-oxoglutarate dehydrogenase complexes may well have identical primary structures. This proposition is now being investigated.

It is interesting that the two cysteine residues forming the disulphide bridge in the active site of lipoamide dehydrogenase are separated in the primary structure by only four residues. This kind of proximity may turn out to be common to enzymes of this type, since in thioredoxin [14] and thioredoxin reductase [15,16] the comparable disulphide bridges are formed by cysteine residues only two residues apart. A possible explanation could be the need to maintain a degree of structural rigidity in the active site: alternate oxidation and reduction of a disulphide bridge that includes a large loop of primary structure might be less acceptable for the purpose (cf. [13]) since the tertiary structure of proteins is more easily disturbed than is local secondary structure. Even a small adventitious disturbance could be sufficient to destroy the efficacious arrangement of the reacting thiol groups. It may also be significant, in so far as knowledge of this extended portion of primary structure allows one to judge, that the active site of lipoamide dehydrogenase appears to be relatively apolar [13]. This would be in keeping with its substrate, enzyme-bound lipoic acid [17].

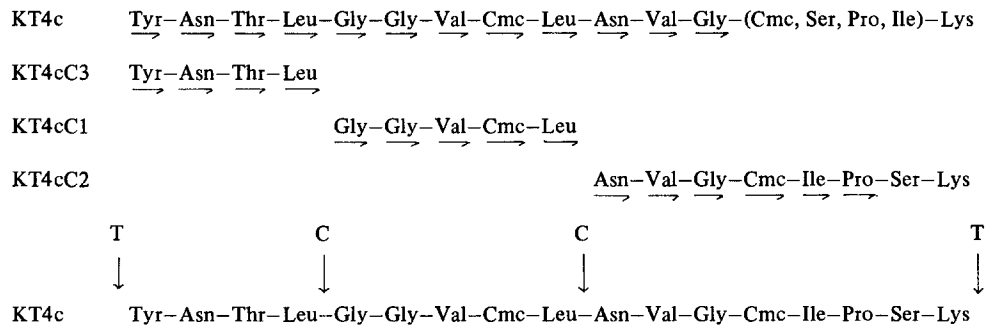

Fig. 1. The amino acid sequences of peptides KT4c, KT4c, KT4cC1, KT4cC2 and KT4cC3. → Denotes a residue established by the dansyl-Edman degradation, T ↓ and C ↓ indicate positions of cleavage by trypsin and chymotrypsin, respectively.

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