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ISOLATION, CHARACTERIZATION AND PARTIAL SEQUENCING OF CYSTINE AND THIOL PEPTIDES OF PIG HEART LIPOAMIDE DEHYDROGENASE

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SUMMARY

Pig heart lipoamide dehydrogenase (EC 1.6.4.3) contains ten half-cystines (as cysteic acid) per mole of enzyme bound FAD. Two of these are linked in an intrachain cystine which acts in concert with the flavin during catalysis. A peptic peptide containing this active center disulfide has been isolated and shown to have the sequence: Glu–Thr–Leu–Gly–Gly–Thr–Cys–Leu–Asn–Val–Gly–Cys–Ile–Pro–Ser (Lys, Ala, Leu). The enzyme also contains seven titratable thiols when either 5,5′-dithiobis (2-nitrobenzoic acid) or iodoacetate is used as titrant. Tryptic peptides containing alkylated thiols have been isolated and characterized by amino acid composition and by their positions in two-dimensional chromatography–electrophoresis. On the basis of map position and composition, the peptides containing thiols can be distinguished from one another. The results are compared with recent data of Brown and Perham (Brown, J. P. and Perham, R. N. (1974) Biochem. J. 138, 505–512) on the compositions and partial sequences of tryptic–chymotryptic peptides containing half-cystines. The combined data associate nine of the ten half-cystines with unique compositions.

INTRODUCTION

The subunit structure of pig heart lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) has been investigated by Massey et al. [1]. The enzyme contains two polypeptide chains which are identical or highly similar and two molecules of tightly bound FAD. The molecular weight of the enzyme has been found to lie between 100 000 and 114 000. Each polypeptide chain was shown to contain ten half-cystines. Two of these are linked in a cystine which acts in concert with the flavin during catalysis. Of the remaining eight half-cystines, only seven can be alkylated by iodoacetate in 5 M guanidine and in the absence of reductant [2]. For this reason we wished to redetermine the half-cystine content of the enzyme using

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TPCK, L-(tosylamido-2-phenyl)-ethylchloromethyl ketone; DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; dansyl, 5-dimethylaminonapthalene-1-sulfonyl.

more recent methods. We also wished to examine the reactivity of the enzyme thiols with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).

The sequence of the peptide containing the active center disulfide is of particular interest because the sequence of the corresponding peptic disulfide peptide from the prokaryote *Escherichia coli* lipoamide dehydrogenase is known from work done in this laboratory [3] and in Perham's laboratory [4]. The comparison of prokaryote and eukaryote sequences should afford insights into the possible origin of this mitochondrial enzyme.

We also wished to identify the thiol residues on the enzyme. Oxidation of thiol residues to a disulfide by cupric ion has been shown to lead to inactivation of the enzyme [5, 6] as has alkylation of native enzyme with iodoacetamide [7]. These results suggest that thiol residues may be located at or near the active center of the enzyme. If the particular thiol residues involved in these modifications are to be identified, it is necessary to have a means of distinguishing one thiol from another. We decided that a preliminary goal would be the location of alkylated thiols in tryptic peptides on peptide maps, and the determination of the amino acid composition of these peptides. Preliminary reports of the sequence of the cystine peptide [2] and the composition of certain of the thiol peptides [8] have been presented. While this work was in progress further data on some of the thiol-containing peptides has appeared [9].

MATERIALS AND METHODS

Purified pig heart lipoamide dehydrogenase was obtained from either Boehringer or Sigma. The enzyme was further purified on calcium phosphate gel-cellulose, essentially as described for *E. coli* lipoamide dehydrogenase [10]. Pure enzyme has an $A_{280\ nm}/A_{455nm}$ ratio of 5.35 [20] and only fractions with ratios between 5.35 and 5.40 were used for structural work.

Reagents for preparation and sequencing of the peptic disulfide peptide were those given previously [3]. A grade, salt-free crystallized trypsin and chymotrypsin were obtained from Calbiochem. All pyridine used for chromatography and electrophoresis was redistilled over ninhydrin.

Reaction of sulfhydryl groups with DTNB. The sulfhydryl content of pig heart lipoamide dehydrogenase was determined in solutions containing 0.4 mM DTNB in 5 M guanidine·HCl, 3 mM in EDTA and 0.07 M in buffer, Tris–HCl, adjusted to pH 8.0; corrections were made for the absorbance at 412 nm of the FAD released from the enzyme. Enzyme aliquots were chosen such that the ratio of DTNB to flavin added was always at least 100:1. The extinction coefficient for the thionitrobenzoate anion at 412 nm was reported by Beutler et al. [11], to be 13 600 $M^{-1}\cdot cm^{-1}$, and this value was confirmed in our laboratory and used for all calculations of thiol content.

Amino acid analysis of pig heart lipoamide dehydrogenase. The methods used in determining the amino acid composition of the unmodified pig heart lipoamide dehydrogenase were identical to those described by Williams, Jr et al. [10]. Tryptophan was determined by a modification of the method of Spies and Chambers as described by Harrison and Hofmann [12]. Half-cystine was measured in separate analyses as cysteic acid after oxidation of the protein with a large excess of dimethylsulfoxide by the method of Spencer and Wold [13].

Isolation, purification and sequencing of the peptic disulfide peptide. The methods

used in the isolation of the cystine peptide from pig heart lipoamide dehydrogenase were essentially the same as those used previously in the isolation of the peptide from *E. coli* lipoamide dehydrogenase [3]. The sulfhydryl groups of the heart enzyme were reacted with *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM), and the enzyme was digested with pepsin. The peptic digest was chromatographed on Sephadex G-25, and the disulfide containing fractions were chromatographed on SE-Sephadex C-25. The disulfide peptide was rechromatographed on SE-Sephadex C-25 which had been pre-equilibrated with 0.05 M pyridine acetate, pH 2.5. After washing the column with 100 ml of the starting buffer the disulfide peptide was eluted using a gradient of 300 ml of starting buffer and 300 ml of 2.0 M pyridine acetate, pH 5.0. Amino acid composition of this peptide indicated that it was uncontaminated.

Mobility of the peptide was determined before and after oxidation with performic acid by electrophoresis at pH 6.5 accompanied by a standard mixture of amino acids. The oxidized peptide was sequenced using manual Edman degradation, according to the previously described procedure [3]. Another portion of the purified, oxidized peptide was subjected to carboxyl-terminal analysis by hydrazinolysis [14].

In preparation for thin-layer chromatography, the phenylthiazolinones released during Edman degradation were converted to phenylthiohydantoins by the method described by Pataki [15]. The phenylthiohydantoins were chromatographed on activated Silica Gel G plates prepared as described by Pataki [15] and the chromatographic solvent was chloroform–methanol (9:1, v/v).

Protocol for anaerobic experiments. The enzyme was placed in an anaerobic cuvette [16] and appropriate reagents were placed in the sidearms. The solution was degassed with six alternating cycles of vacuum and nitrogen. The nitrogen gas was purified by passage over a heated column (190 °C) containing catalytic copper (BTS catalyst made by Badische Anilin und Soda Fabrik).

Alkylation of thiols. 200–500 nmoles of enzyme (with respect to bound FAD) were dissolved in 2 ml of 7.5 M guanidine·HCl, 4.5 mM in EDTA and 0.1 M in Tris–HCl, pH 8.0. If reduction of the disulfide peptide was desired, a 5-fold excess of dithiothreitol over enzyme-bound FAD was added and the solution incubated for 30 min at room temperature. A 0.1 M solution of iodoacetate in 1 M unneutralized Tris was prepared with a specific radioactivity of 2 Ci/mole. An amount of iodoacetate stoichiometric with the total thiol content of the enzyme solution (including any dithiothreitol present) was added to the enzyme solution, anaerobically, in the apparatus described above, and the solution was incubated in the dark for 1 h at room temperature. The solution was then dialyzed against 1% ammonium bicarbonate. The extent of alkylation was determined by amino acid analysis of *S*-carboxymethylcysteine, using strict anaerobiosis during hydrolysis as recommended by Moore and Stein [17].

Tryptic digestion of alkylated enzyme and preparation of peptide maps. The dialyzed enzyme was digested with trypsin in 1% ammonium bicarbonate at 37 °C for 20 h with constant stirring, and aliquots of trypsin equivalent to 2% (by wt) of enzyme were added at the start of the incubation and after 1 and 3 h. 25–50 nmoles of the tryptic digest were dried in an Evapomix at 30 °C, dissolved in glass-distilled water, and applied to a sheet of Whatman No. 3 chromatography paper. Descending chromatography in butanol–acetic acid–water–pyridine (15:3:12:10, by vol.) was carried out for 20 h. The paper was dried at 32 °C for 24 h and subjected to electro-

phoresis at right angles to the direction of chromatography. Electrophoresis at pH 6.5 was performed in a Savant electrophoresis tank at 3000 V for 1 h. The paper was dried at 32 °C for 1 h and a radioautogram prepared by exposure for 24–72 h.

Isolation, purification and analysis of peptides. Radioactive peptides were eluted from paper with 0.1 M NH_4OH. The peptides from several maps were pooled and 100–150 nmoles of peptide (determined by radioactivity) were applied to strips of Whatman No. 3 chromatography paper for electrophoresis at pH 3.5 and/or 1.9. Radioautograms were used to locate the radioactive alkylated thiol peptides. Prior to amino acid analysis, the purified peptide was hydrolyzed for 24 h in 6 M HCl containing 1 % β-mercaptoethanol, dried in the Evapomix and dissolved in citrate–HCl buffer, pH 2.2. Any β-hydroxyethyldisulfide was removed by centrifugation. Just prior to application to the analyzer, the radioactivity of an aliquot of the sample in the citrate–HCl buffer was measured to provide an exact correlation of moles of label per mole of peptide hydrolysate. Radioactivity was measured in a Hewlett–Packard liquid scintillation spectrometer.

The amino acid analyses were carried out on a Beckman 120B amino acid analyzer equipped with long path-length cells. Samples were injected automatically from pre-calibrated loops. The single column, three buffer system of the Durrum Chemical Corp. was used. DC-1A resin, ammonia traps and the buffer concentrates were obtained from Durrum.

Amino-terminal analysis of the thiol peptides was performed according to Gray [18] by dansylation, 16–20 h acid hydrolysis, and identification of the dansyl amino acid derivatives by thin-layer chromatography on Cheng-Chin polyamide plates.

Chymotryptic digestion of isolated peptides was performed according to Maita and Konigsberg [19].

RESULTS

Table I shows the results of amino acid analysis of pig heart lipoamide dehydrogenase. The molecular weight per subunit determined by amino acid analysis is 51 000, in good agreement with the value of 50 000–57 000 cited by Massey [20]. We have confirmed the value of ten half-cystines per FAD found by Massey et al. [1] using dimethylsulfoxide as the oxidant, and our values for other residues are in reasonable agreement with earlier analyses [1, 21, 22].

When the native enzyme is denatured in 5 M guanidine·HCl, 6.9–7.0 thiol groups react with DTNB. This is in agreement with results previously obtained on alkylation of the denatured protein with iodoacetic acid [2] which gave values of 6.4–6.9 *S*-carboxymethylcysteines per FAD, determined by amino acid analysis of the hydrolyzed alkylated protein. If the enzyme is reduced with dithiothreitol in 5 M guanidine prior to alkylation with iodoacetate, 8.3 *S*-carboxymethylcysteines are formed per mole of FAD.

The methods used in the isolation of the pig heart lipoamide dehydrogenase cystine peptide reflect the need to prevent thiol–disulfide interchange. Reaction of the sulfhydryl groups with DDPM and digestion with pepsin allow the maintenance of low pH during the production of the disulfide peptide. The chromatographic procedures are also carried out between pH 2 and 5.

TABLE I

AMINO ACID COMPOSITION OF LIPOAMIDE DEHYDROGENASE

Cys-(O_3H) and Try determined separately (see the text); Thr and Ser, values extrapolated to zero time of hydrolysis; Pro, determined in a separate experiment after oxidation with dimethylsulfoxide to remove cysteine which coelutes with proline.

Amino acid	Moles per mole of FAD found after hydrolysis			Proposed
	24 h	48 h	72 h	
Lys	35.0	36.6	36.5	36
His	10.1	10.7	11.1	11
Arg	13.9	14.3	14.2	14
Cys-(O_3H)	10.1	10.1	10.0	10
Asp	44.0	44.0	43.3	44
Thr	25.5	24.7	23.5	26
Ser	21.2	18.6	15.6	24
Glu	47.3	48.4	47.2	47
Pro	18.0	—	—	18
Gly	54.1	52.9	53.2	53
Ala	46.6	44.3	46.6	46
Val	41.7	44.7	44.2	44
Met	9.1	9.0	9.5	9
Ile	35.4	35.6	35.6	36
Leu	31.3	30.3	31.1	31
Tyr	9.0	7.8	8.3	8
Phe	14.9	14.3	14.4	15
Try				2
Total residues				474

Amino acid analysis of the purified peptide both before and after performic acid oxidation indicated the following composition: Lys, Cys_2, Asp, Thr_2, Ser, Glu, Pro, Gly_3, Ala, Val, Ile, $Leu_{2.5}$. Electrophoresis at pH 6.5 of the peptide before oxidation indicated a neutral peptide, so that either the aspartyl or glutamyl residue must be present as the amide. The mobility of the peptide on electrophoresis at pH 6.5 was also measured after performic acid oxidation, and by comparison with the standard plots of Offord [23] indicated a net charge of −2.

Table II shows the molar ratios for each step of the Edman degradation, with the italicized residues averaged as the bases for calculation of molar ratios. The residues in parentheses are those whose ratio has dropped with that degradation step. There was insufficient material to obtain reliable analyses after Edman degradation steps No. 16 and No. 17, but only lysine, alanine and leucine remain unaccounted for. For this reason hydrazinolysis was performed to determine the carboxyl-terminal composition of the peptide. The results show that 54% of the carboxyl-terminal residues were alanine and 46% were leucine. Since pepsin may cleave on either side of leucyl residues [24], such a finding was not unexpected, and it explains the fractional value for leucyl residues found in the peptide (see Table II). The peptic disulfide peptide apparently consists of a mixture of two peptides which are identical except for the presence or absence of leucine in the carboxyl-terminal position.

Assignment of the amide in the disulfide peptide was made by thin-layer

TABLE II

SUMMARY OF MOLAR RATIOS OF EDMAN DEGRADATIONS ON PERFORMIC ACID-OXIDIZED DISULFIDE PEPTIDE

Residues averaged as the base for calculation are italicized in each step; the residue which has dropped in ratio in a given step is enclosed in parentheses.

Residue	Initial	Edman step number														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Cys(O_3H)	1.82	1.5	1.4	1.6	1.4	1.6	1.6	(1.2)	1.2	1.2	1.3	1.3	(1.1)	0.9	1.0	0.9
Asp	1.04	*1.0*	*1.0*	*1.0*	*0.9*	*1.0*	*1.1*	*1.1*	*1.0*	(0.5)	0.4	0.4	0.5	0.5	0.5	0.5
Thr	1.87	1.6	(1.1)	*1.0*	*1.0*	*1.0*	(0.5)	0.5	0.4	0.4	0.4	0.5	0.5	0.5	0.5	0.5
Ser	0.89	0.9	1.5	1.0	0.8	0.9	0.9	0.9	1.0	0.9	0.9	0.9	1.0	0.9	0.9	(0.6)
Glu	0.97	(0.2)	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.2	0.3
Pro	0.93	0.7	0.7	*1.1*	0.8	*0.9*	*0.9*	*1.0*	*0.9*	*1.0*	*1.0*	*1.2*	*1.1*	*1.0*	(0.4)	0.5
Gly	2.96	2.6	2.9	2.8	(2.0)	(1.6)	1.5	1.5	1.5	1.5	1.5	(0.9)	1.1	1.1	1.0	1.1
Ala	1.10	*0.1*	*1.1*	*1.0*	*1.0*	*1.1*	*1.0*	*1.0*	*1.1*	*1.1*	*1.0*	*0.9*	*0.9*	*1.1*	*1.0*	*1.0*
Val	0.94	*0.1*	*0.9*	*1.0*	*1.1*	*1.1*	*1.1*	*1.1*	*1.0*	*1.1*	(0.5)	0.5	0.5	0.5	0.6	0.4
Ile	0.99	0.8	*0.9*	*0.9*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	*1.0*	(0.6)	0.6	0.5
Leu	2.65	2.2	2.3	(1.7)	1.6	1.7	1.9	1.7	(1.2)	1.2	1.1	1.2	1.1	1.1	1.0	1.0
Lys	0.96															

chromatography of the phenylthiohydantoins prepared from the phenylthiazolinones released during Edman degradation steps No. 1 and No. 9. The first residue had the mobility of the phenylthiohydantoin of glutamate, and the ninth residue had the mobility of the phenylthiohydantoin of asparagine. The sequence of the cystine containing peptide deduced from the data presented is given below:

Glu–Thr–Leu–Gly–Gly–Thr–Cys–Leu–Asn–Val–Gly–Cys–Ile–Pro–Ser(Lys, Ala, Leu)

A radioautogram of a typical peptide map of enzyme alkylated with iodo-[$^{14}C_1$]acetate in the absence of added reductant and then digested with trypsin is shown in Fig. 1A. Nine radioactively labelled peptides can be seen, one cationic, four neutral and four anionic peptides. Fig. 1B shows the radioautogram of a peptide map of the tryptic digest of enzyme which was first alkylated with unlabelled iodoacetate in the absence of reductant, and then was reduced with dithiothreitol, and

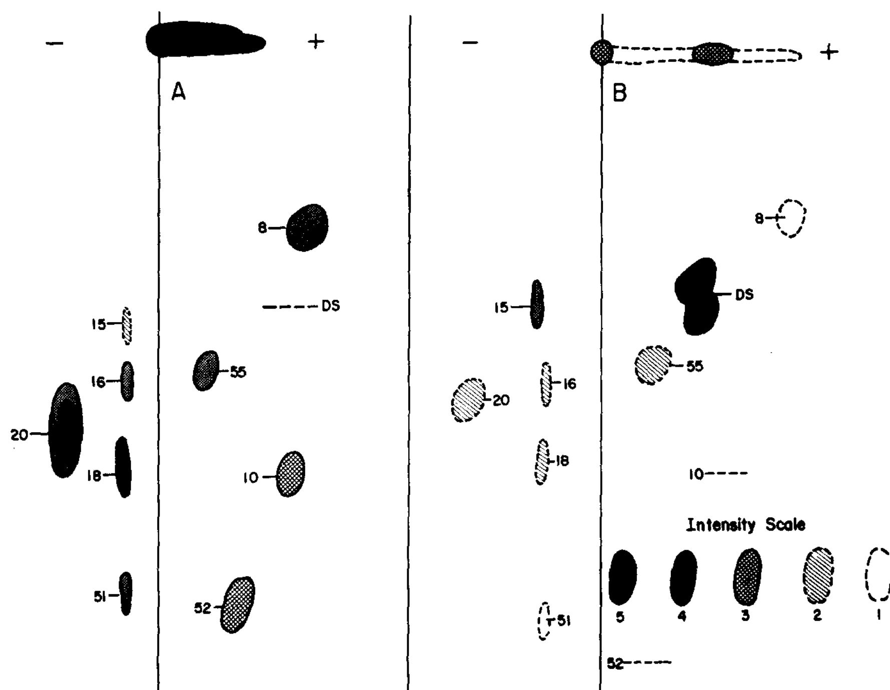

Fig. 1. The left half (A) of this figure shows the radioautogram of a peptide map of a tryptic digest of heart lipoamide dehydrogenase which had been alkylated with iodo[$^{14}C_1$]acetate in the presence of guanidine and then reduced and realkylated with unlabelled iodoacetate. Descending chromatography was performed in the vertical dimension and pH 6.5 electrophoresis in the horizontal dimension, as described in the text. The origin is at the top on the vertical line. The electrophoretic cathode was located to the left, and the anode to the right of the origin. The right half (B) of this figure shows the radioautogram of a similar peptide map prepared with a tryptic digest of enzyme alkylated with unlabelled iodoacetate in the presence of guanidine, and then reduced and realkylated with iodo[$^{14}C_1$]acetate. The orientation of the peptide map is the same as in the left half of the figure. Identical amounts of digested protein as determined by amino acid analysis (30 nmoles) were used for the two peptide maps.

realkylated with iodo[$^{14}C_1$]acetate. In this manner only the peptide(s) containing thiols originally in disulfide bonds should be radioactively labelled. The appearance of a prominent new peptide, marked Peptide DS, is apparent. A second peptide, Peptide 15, is more prominent in Fig. 1B than in Fig. 1A. The appearance of small amounts of the other peptides on the radioautogram of Fig. 1B probably indicates that these peptides are not completely modified during alkylation of enzyme with an intact disulfide bond. (Identification of the labelled peptides is greatly assisted by staining the entire map with cadmium–ninhydrin, since the characteristic colors and grouping of the peptides are quite reproducible from one map to another, even though small differences in the chromatography or electrophoresis of a given peptide may occur.)

The peptides from such maps can be eluted from the paper, purified by re-electrophoresis at pH 3.5 and/or pH 1.9, and subjected to amino acid analysis. The results of analysis of these radioactively labelled peptides are shown in Table III. All the peptides seen on the radioautograms have their label as *S*-carboxymethylcysteine, as can be seen by a comparison of the *S*-carboxymethylcysteine content determined by amino acid analysis and the concentration of peptide as determined by radioactive counts in the peptide hydrolysate applied to the analyzer.

TABLE III

AMINO ACID COMPOSITIONS OF ^{14}C-LABELLED, ALKYLATED TRYPTIC PEPTIDES

The table lists molar equivalents of amino acid residues present. The base for calculation of molar eqivalents was determined by residue averaging of italicized residues and comparison with the radioactivity of the sample analyzed.

Amino acid	Peptide									
	20	18	51	16	8	10	55	52	DS	15
Lys							*1.1*		*1.0*	*1.1*
His	1.8									
Arg	*1.0*	*0.9*	*0.9*	*1.0*	*1.0*	*1.0*				
CM-Cys	*0.9*	*1.0*	*0.9*	0.7	0.8	*1.0*	*1.0*	*1.0*	0.7	0.7
Asp					*1.9*	1.7		1.4	*1.2*	1.4
Thr	*1.1*						*0.9**		*1.0*	
Ser	*1.1*				1.3	*1.9*		*1.2*	1.3	1.2
Glu	*1.0*				*2.0*	*2.8*	1.3		1.2	0.4
Pro	*1.0*							*1.0*	0.6	0.6
Gly		*1.2*	1.6	*2.0*	1.8	*2.1*			2.3*	1.5
Ala	1.8				2.1	*3.1*			0.6	0.6
Val	*1.0**	*1.0**	*1.2*				*1.0*	*2.0*	*1.0*	*1.1*
Met										
Ile		*1.0*	*1.0*	*1.0*	*1.1*	*1.1*	*0.9*	*0.8*	*0.8*	*0.8*
Leu	*0.9*		*1.1**			*1.2*			*1.0*	
Tyr						*1.0**		*1.0*		
Phe	*1.1*									
nmoles by residue averaging	10.1	11.8	7.1	6.8	10.1	10.0	10.4	7.8	7.2	7.8
nmoles by radioactivity	10.1	11.4	7.1	7.5	8.4	10.3	10.8	7.5	7.2	5.7

* Shown to be N-terminal residues by dansylation.

The sequence of Peptide 18 was determined in our laboratory by Dr Eric Jones, using the dansyl-Edman method [25]. The sequence was found to be: Val–Cys(Cm)–Ile–Gly–Arg. We had noticed that the remaining two neutral peptides, Peptides 16 and 51, also appeared to be very small and had very similar mobilities versus serine on electrophoresis at pH 1.9; the R_F of Peptide 18 was 1.09, that of Peptide 51 was 1.03, and that of Peptide 16 was 1.14. Peptides 16 and 51 are isolated in very low yield (less than 10%) and we have been unable to purify them completely. However, the composition of Peptide 51, combined with the fact that Offord plots [23] indicated a molecular weight only slightly greater than that of Peptide 18, suggested that Peptide 51 might differ from Peptide 18 only by the presence of an additional amino-terminal leucine. Dansylation of Peptide 51 showed leucine to be amino-terminal. Thus we suspect that Peptide 18 is formed from Peptide 51 by chymotryptic cleavage to remove the leucine. Amino acid analysis of Peptide 16, combined with the fact that Offord plots indicate a peptide slightly smaller than Peptide 18, suggest that this peptide will prove to have a related sequence.

The compositions of Peptides 8 and 10 suggested that Peptide 8 might be derived from Peptide 10 by chymotryptic cleavage. This was verified by preparing a peptide map of a chymotryptic digest of Peptide 10. The radioactivity now appeared in the position expected for Peptide 8, with only traces of radioactivity appearing in the original position.

Peptides 20, 18–51, 55, 52 and 8–10 clearly represent unique thiol peptides. Thus, we have identified five thiol peptides out of the total of seven titratable cysteine-containing peptides in the polypeptide chain.

The appearance of Peptide DS in the radioautogram of Fig. 1B suggests that it is the disulfide peptide produced by tryptic cleavage and the composition of Peptide DS agrees very well with that expected from the sequence of the peptic peptide containing the active center disulfide.

The appearance of Peptide 15 in the radioautogram of Fig. 1B and its similarity in amino acid composition to Peptide DS suggest that it is a derivative of Peptide DS. A peptide of this composition could be created by chymotryptic cleavage of Peptide DS on the carboxyl-terminal side of the leucyl residue located between the two cysteines. Chymotryptic cleavage during digestion with trypsin is not unexpected since we used long digestion times (20 h) and high trypsin concentrations (6%) in order to enhance digestion, and found that L-(tosylamido-2-phenyl)ethylchloromethyl ketone (TPCK) treated trypsin led to much poorer digestions of this enzyme. To test the hypothesis that Peptide 15 is derived by chymotryptic cleavage of Peptide DS, the isolated ^{14}C-labelled Peptide DS was subjected to chymotryptic digestion, and the digest was applied to paper and a peptide map prepared by chromatography and then electrophoresis at pH 6.5. A faint radioactive spot was seen at the normal position of Peptide DS on the map, probably representing undigested Peptide DS. In addition a radioactive spot appeared in the neutral lane, in the position expected for Peptide 15. A third radioactive spot appeared which may well represent the other portion of Peptide DS. The identification of Peptide 15 as a chymotryptic cleavage product of the reduced, ^{14}C-labelled disulfide peptide explains its appearance in Fig. 1B.

DISCUSSION

While this manuscript was in preparation, Brown and Perham [9] published data on the sequences of tryptic peptides from the active center and complete or partial sequences of peptides containing six of the seven reactive thiols from a tryptic–chymotryptic digest. Our previously published [8] sequence of Peptide 18, and compositions of Peptides 20 and 55 were confirmed by their work. Table IV demonstrates the probable relationships between the peptide compositions shown in Table III and the thiol peptides reported by Brown and Perham [9]. Thus we are able to identify five unique thiols from the compositions of the tryptic peptides while Brown and Perham are able to identify six unique thiols. With the combined data of the two laboratories, each of the seven reactive thiols can be identified. We do not see peptides corresponding to the chymotryptic–tryptic Peptides DTCla and DTClb2 isolated by Brown and Perham [9].

TABLE IV

SEQUENCES AROUND THE REACTIVE THIOLS

Composition and sequence data from the present work and from the work of Brown and Perham [9] are compared. ⇀, a residue placed by the dansyl-Edman or the subtractive-Edman procedures. R_F refers to peptide mobility on electrophoresis at pH 6.5 relative to the mobility of aspartic acid (−1.0).

Peptide	R_F	Sequence
51	0.00	Leu (Val, Cys (Cm), Ile, Gly) Arg
18	0.00	Val–Cys (Cm)–Ile–Gly–Arg
DTC4a	0.00	Val–Cys (Cm)–Ile–Gly–Arg
20	+0.14	Val (Cys (Cm), His, Ala, His, Pro, Thr, Ser, Glx, Ala, Leu, Phe) Arg
DTC2n2	+0.15	Val–Cys (Cm)–His–Ala–His–Pro (Thr, Ser, Glx, Ala, Leu, Phe) Arg
55	−0.23	Thr (Val, Cys (Cm), Ile, Glx) Lys
DTC2b	−0.27	Thr–Val–Cys (Cm)–Ile–Glu–Lys
10		Tyr (Ser, Gly, Ala, Leu, Gln, Gly, Asn, Gly, Ala, Ser, Cys (Cm), Glu, Asp, Ile, Ala) Arg
8	−0.51	(Gln, Gly, Asn, Gly, Ala, Ser, Cys (Cm), Glu, Asp, Ile, Ala) Arg
DTC2a2	−0.52	Gly–Ala–Ser–Cys (Cm)–Glu–Asp–Ile–Ala–Arg
52	−0.30	(Cys (Cm), Asp, Ser, Pro, Val_2, Ile, Tyr) No corresponding peptide
DTCla	−0.87	Ala–Glx–Asx–Glx–Gly–Ile (Cys (Cm), Glx, Gly, Val, Met) No corresponding peptide
DTClb3	−0.63	Ala–Gly–Val–Ile–Thr–Cys (Cm)–Asp–Val–Leu–Leu No corresponding peptide

Both laboratories have been unable to react the eighth half-cystine with thiol reagents. In the course of these studies we have been consistently perplexed by this failure of one-half cystine to be alkylated. The evidence for a total of ten half-cystines per enzyme bound FAD rests on the amino acid analysis of oxidized enzyme. Since the flavin is very firmly bound, exact quantitation of protein is readily obtained. Indeed, many preparations of enzyme have been analyzed and the ratio of flavin to the six most stable residues is invariant. Each polypeptide chain contains a single disulfide and seven reactive thiols, leaving one half-cystine unaccounted for. Even enzyme which has been reduced with dithiothreitol in 5 M guanidine does not alkylate completely, although such conditions should reduce any disulfide bonds present in the molecule. The non-reactivity of the eighth half-cystine can not, therefore, be explained simply by its involvement in a second disulfide. Brown and Perham [9] have shown that pig heart lipoamide is monomeric on sodium dodecylsulfate disc gel electrophoresis without prior reduction, indicating that the eighth half-cystine is not involved in an interchain disulfide bond.

Table V compares the sequence and composition data for peptides from the active center disulfide region obtained by us and by Brown and Perham [9].

Brown and Perham were able to show that the disulfide bond at the active center was originally located in a large fragment DT2a, (not shown) but due to the presence of three thiols in the reduced alkylated peptide they were unable to specify which two cysteines were actually involved in the active center disulfide.

TABLE V

SEQUENCES IN THE ACTIVE CENTER DISULFIDE REGION

Composition and sequence data from the present work and from the work of Brown and Perham [9] are compared HPDS, the peptic peptide containing the disulfide region. Where the peptide has been isolated as the disulfide oxidized with performic acid and sequenced, this has been indicated as a disulfide bond. Sequences from the analogous region of the *E. coli* enzyme are also included [3, 4].

Peptide	R_F	Sequence
HPDS		S————S (Cys–Cys) Glu–Thr–Leu–Gly–Gly–Thr–Cys–Leu–Asn–Val–Gly–Cys–Ile–Pro–Ser(Lys,Ala,Leu)
DTC2n1 (ref. 9)	0.00	Cm (Cys) Asn–Val–Gly–Cys–Ile–Pro–Ser–Lys
DT2aT1 (ref. 9)	−0.38	Cm, Cm (Cys, Cys) Asx–Glx–Thr–Leu–Gly–Gly–Thr(Cys,Leu,Asn,Val,Gly,Cys,Ile,Pro,Ser)Lys
EcPTDS (ref. 3)		S————S (Cys–Cys) Val–Cys–Leu–Asn–Val–Gly–Cys–Ile–Pro–Ser–Lys
KT4c (ref. 4)	−0.20	Cm, Cm (Cys, Cys) Tyr–Asn–Thr–Leu–Gly–Gly–Val–Cys–Leu–Asn–Val–Gly–Cys–Ile–Pro–Ser–Lys

Our peptic peptide was isolated as the disulfide peptide and since it contains only two half-cystines, the residues forming the disulfide bond are unambiguously determined. It must be noted that this sequence differs from that previously published by us [2] in the position of the lysine. Subsequent to the publication of Brown and Perham [9], we rechecked our original data finding an error and have since resequenced the relevant portion; the sequences from the two laboratories are now in agreement.

The sequence of the active center disulfide peptide from *E. coli* lipoamide dehydrogenase, based on the data of Burleigh and Williams [3] and of Brown and Perham [4] is shown at the bottom of Table V. In the overlapping region, 14 of the 17 overlapping residues are homologous. This is the longest homology thus far reported for analogous peptides isolated from enzymes of a prokaryote and eukaryotic mitochondria. Thus this portion of the polypeptide chain has been rigidly conserved during the long period of evolution subsequent to the divergence of these two species from a presumed common ancestor. This rigid conservation is perhaps not surprising given the rather demanding catalytic requirements for close interaction, initially between lipoamide (covalently bound to the transacetylase) and the redox active disulfide; then between the disulfide and the flavin; and finally between the FAD–thiol complex and the pyridine nucleotide.

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