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A Comparison of Thiol Peptides of Heart Mitochondrial Malate Dehydrogenases from Pig, Chicken, and Tuna*

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ABSTRACT: The thiol groups of mitochondrial malate dehydrogenases (EC 1.1.1.37) from pig, chicken, and tuna hearts were alkylated with 1-[^{14}C]iodoacetate in 8 M urea, and the alkylated enzymes digested with trypsin and with mixed trypsin–chymotrypsin. Peptide mapping of the digests revealed a carboxymethylcysteine-containing peptide which appeared to be the same in all three malate dehydrogenases in its position on the peptide maps, its migration during electrophoretic purification at pH 3.5, and its amino acid composition. This peptide common to the three malate dehydrogenases contained one residue each of lysine, [^{14}C]carboxymethylcysteine, threonine, serine, glutamic acid (or glutamine), proline, and isoleucine when isolated from tryptic peptide maps. When isolated from tryptic–chymotryptic maps it contained all the same residues except for the absence of threonine. A second and larger peptide containing carboxymethylcysteine also appeared to be common to the tryptic peptide maps of all three enzymes and contained the same residues as the first peptide plus one additional residue each of threonine or serine, glycine, alanine, isoleucine, leucine, and possibly proline. The first peptide may be a chymotryptic fragment of the second, and both appear to represent a thiol-containing region in mitochondrial malate dehydrogenases which is conserved during evolution. The effect of iodoacetate on native chicken heart mitochondrial malate dehydrogenase in the presence and absence of substrates and coenzymes has been studied. A tryptic digest of the enzyme after total thiol alkylation in 8 M urea has been fractionated on a cation-exchange column, and the carboxymethylcysteine-containing peptides purified by paper electrophoresis and anion-exchange column chromatography. The peptides so isolated have been compared to those obtained from the peptide maps. The use of comparative peptide mapping in the identification of functionally essential regions in proteins is discussed.

The involvement of thiol groups in the operation of NAD-linked dehydrogenases has been established for several enzymes including glyceraldehyde-3-P dehydrogenase (Harris *et al.*, 1963), lactate dehydrogenase (Gruber *et al.*, 1962; DiSabato and Kaplan, 1963; Dube *et al.*, 1963), and alcohol dehydrogenase (Li and Vallee, 1965). Complete amino acid sequences around essential thiol groups in these enzymes have also been determined (Harris *et al.*, 1963; Fondy *et al.*, 1965; Holbrook and Pfleiderer, 1965; Gold and Segal, 1965; Holbrook *et al.*, 1967; Mella *et al.*, 1968; Harris, 1964; Li and Vallee, 1964).

A number of studies have implicated thiol groups in the catalytic mechanism of malate dehydrogenases. Green (1936) found that pig heart malate dehydrogenase was inhibited by iodoacetamide and similar findings were reported by Grimm and Doherty (1962) who showed that iodoacetamide-treated pig heart mitochondrial malate dehydrogenase bound substantially less NADH than did the native enzyme. Other workers (Wolfe and Neilands, 1956; Siegel and Englard, 1962; Devenyi *et al.*, 1966b; Kitto and Kaplan 1966; Seguin and Kosicki, 1967; Thorne and Kaplan, 1966; Siegel, 1967) have shown the sensitivity of malate dehydrogenases to thiol reagents such as *p*-hydroxymercuribenzoate and 5,5′-dithiobis-(2-nitrobenzoate). In general, the reaction with sulfhydryl reagents is sluggish in the absence of denaturing agents such as urea, indicative of buried thiol groups, and it is apparent that not all of the sulfhydryl groups are essential for activity (Siegel and Englard, 1962; Seguin and Kosicki, 1967; Devenyi *et al.*, 1966b; Siegel, 1967; Guha *et al.*, 1968). However, it has not been possible so far to label specifically and to identify an essential thiol region in malate dehydrogenases.

The active-site thiol region in lactate dehydrogenase was identified by Fondy *et al.* (1965) and Fondy and Kaplan (1965) by a structural comparison of thiol peptides obtained from lactate dehydrogenases of several different species widely separated in the evolutionary time scale. The essential thiol region in lactate dehydrogenases from frog, dogfish, and chicken muscle and from bovine and chicken heart showed very similar or identical primary sequences. Mella

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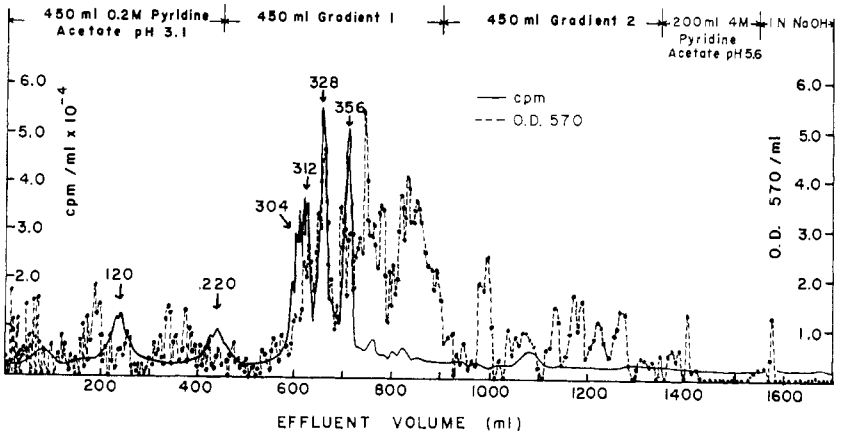

FIGURE 1: Cation-exchange Dowex 50-X2 column (1.2 × 100 cm) chromatography of 90 mg of chicken heart mitochondrial malate dehydrogenase alkylated with 1-[^{14}C]iodoacetate in 8 M urea and containing 14 g-atoms of ^{14}C/mole of enzyme. Numbered peaks are the six major [^{14}C]carboxymethylcysteine-containing peptides that were subjected to further purification and characterization.

et al. (1968) determined the amino acid sequences of the essential thiol peptide from pig muscle and chicken muscle lactate dehydrogenases and found that the sequences were identical with each other and to the sequence determined by Holbrook *et al.* (1967) for the pig heart enzyme. This common sequence is in turn very similar to the essential thiol region in chicken heart lactate dehydrogenase (Fondy *et al.*, 1965; Fondy and Kaplan, 1965) and therefore is also very similar or identical with the essential thiol region in the frog, dogfish, and bovine enzymes mentioned above.

Similarly, the portion of the amino acid sequence of glyceraldehyde-3-P dehydrogenase which contains the essential thiol group is identical in the yeast, rabbit muscle, and pig muscle enzymes (Perham and Harris, 1963) and differs only slightly from these in the enzyme from lobster muscle (Davidson *et al.*, 1967).

It is therefore reasonable to attempt to identify an essential thiol region in the primary sequence of an enzyme by finding a thiol peptide which is particularly strongly conserved during evolution. We have made such an attempt to identify possible essential thiol peptides in mitochondrial malate dehydrogenases by a comparison of thiol peptides obtained from trypsin digests of 1-[^{14}C]carboxymethylated pig, chicken, and tuna enzymes. This approach is complicated by the relatively larger number of thiol peptides in these malate dehydrogenases (six to eight) as compared to lactate dehydrogenases used in the earlier work (one to five).

Devenyi *et al.* (1966a) have previously published an analysis of the thiol peptides obtained from pig heart mitochondrial malate dehydrogenase. We have employed an essentially similar approach on the pig heart enzyme and extended the analysis to thiol peptides eluted from peptide maps of chicken heart and tuna heart mitochondrial malate dehydrogenases in an attempt to identify any thiol peptide which might be common to all three malate dehydrogenases studied. At least one such peptide has been observed, and it and others have been isolated on a preparative scale by ion-exchange column methods and the amino acid compositions established quantitatively.

Methods

Enzymes and Enzyme Assays. The isolations of the mitochondrial malate dehydrogenases including methods of assay have been detailed in previous publications (Kitto and Kaplan, 1966; Kitto and Lewis, 1967; Wolfe and Neilands, 1956; Thorne, 1962; Joyce and Grisolia, 1961).

Peptide Mapping, Specific Staining, and Electrophoresis at pH 3.5. Procedures used were identical with those employed by us in the study of lactate dehydrogenases (Fondy *et al.*, 1965; Pesce *et al.*, 1967; Fondy *et al.*, 1964).

Amino Acid Analysis. Analysis of 24-hr acid hydrolysates was done with a Beckman amino acid analyzer fitted with long-path cuvets and recorder scale expansion. Reliable quantitative analyses were obtained for samples as low as 5 × 10^{-9} mole of peptide hydrolysate per column.

Ion-Exchange Column Chromatographic Separation of Tryptic Peptides of Carboxymethylated Chicken Heart Mitochondrial Malate Dehydrogenase. The cation-exchange Dowex 50-X2 column separation is detailed under Results, and the elution schedule shown in Figure 1. The procedure is that used by Matsubara and Smith (1963). The buffers contained 0.2% thiodiglycol.

Anion-exchange Dowex 1-X2 column purification of peptide fractions followed the procedure of Schroeder *et al.* (1962). A 0.9 × 52 cm column was packed and equilibrated in pH 8.0 buffer (47 ml of pyridine–113 ml of α-picoline–0.5 ml of glacial acetic acid, diluted to 4 l. with water). After sample addition the column was eluted with 30 ml of the pH 8.0 buffer, then with a linear gradient of 38 ml of the pH 8.0 buffer and 38 ml of 0.1 M acetic acid.

Edman Degradation. The Edman method as detailed by Konigsberg and Hill (1962) was used in our work.

Results

Effect of Iodoacetate on Native Chicken Heart Mitochondrial Malate Dehydrogenase. Since it is possible specifically to alkylate an essential thiol group in glyceraldehyde-3-P dehydrogenase (Harris *et al.*, 1963) and in horse liver alcohol dehydrogenase (Li and Vallee, 1964) using iodoacetate, we attempted a similar procedure with malic dehydrogenase from chicken heart mitochondria. A dilute solution of the enzyme (3 × 10^{-10} M) was not affected by incubation in the presence of 0.3 mM iodoacetate at 25° for 10 min in 0.1 M phosphate (pH 7.5). Incubation of the dilute enzyme solution for 30 min with 0.6 mM iodoacetate produced a 40% decrease in activity when compared with enzyme incubated in the absence of

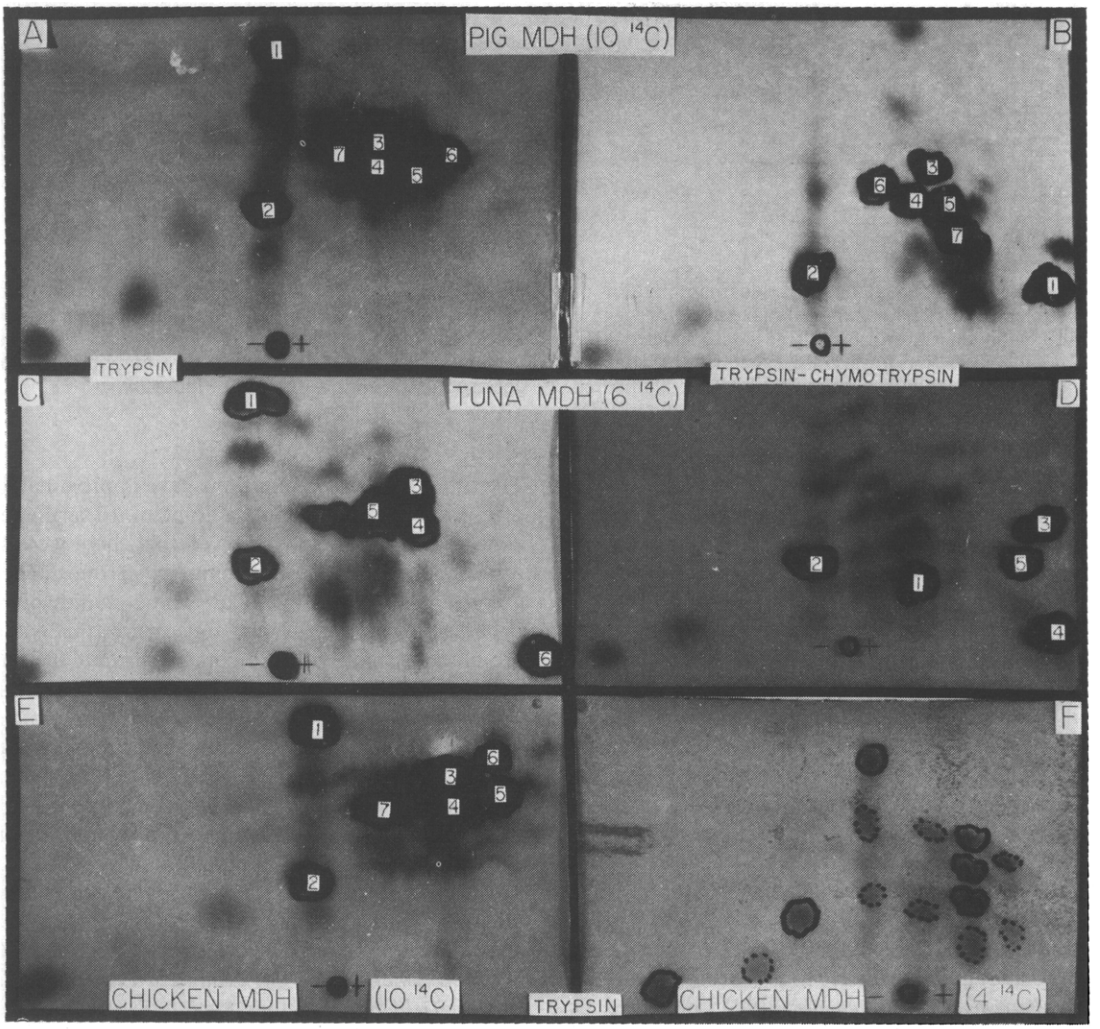

FIGURE 2: Autoradiographs of peptide maps of proteolytic digests of [^{14}C]carboxymethylated heart mitochondrial malate dehydrogenases. Conditions are as in Figure 3. A–E are of malate dehydrogenases alkylated with 1-[^{14}C]iodoacetate at pH 8.2 in 8 M urea. F is of the native chicken heart enzyme 75% inactivated with 1-[^{14}C]iodoacetate at pH 7.5 by multiple freezing and thawing. Amino acid composition of peptides in autoradiographs A through E are presented in Table I. Peptides for which no analysis appears in Table I were recovered from paper in yields too low for meaningful analysis.

iodoacetate. A more concentrated enzyme solution of 1.6 mg/ml (2.4×10^{-5} M) when incubated in 50 mM iodoacetate for 2 hr not only failed to show inhibition, but actually gave a twofold increase over control activity. It thus appeared that any essential thiol groups in chicken heart mitochondrial malate dehydrogenase resemble the essential thiols of lactate dehydrogenases and are not readily alkylated by iodoacetate in the native enzyme (Fondy *et al.*, 1965), rather than those of glyceraldehyde-3-P dehydrogenase or alcohol dehydrogenase which are susceptible to alkylation.

It has been found that essential thiol groups in bovine heart lactate dehydrogenase which are not affected by *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide in the native enzyme are specifically alkylated when the reaction mixture is subjected to freezing and thawing (Gold and Segal, 1965). We therefore attempted freezing and thawing of 2.4×10^{-5} M malate dehydrogenase in 50 mM iodoacetate and observed a 55% inactivation. Multiple freezing and thawing further reduced the activity to 25% of the initial activity. Multiple freezing and thawing of malate dehydrogenase at this concentration in the absence of iodoacetate did not affect the activity. Freezing and thawing of a dilute solution (10^{-10} M) on the other hand produced complete inactivation even in the absence of iodoacetate. Studies by Blonde *et al.* (1966) showed that mitochondrial malate dehydrogenase from pig heart at 10^{-6} M was also stable to freezing and thawing in phosphate buffer in the presence of bovine serum albumin.

In order to determine if the inactivation caused by freezing and thawing in the presence of iodoacetate might involve alkylation of active-site groups in the enzyme, we carried out the reaction in the presence of oxidized or reduced coenzyme separately, and together with oxidized and reduced substrates. NADH and malate, separately or together, did not prevent the 50–70% inactivation of the enzyme caused by freezing and thawing a 2.4×10^{-5} M solution in the presence of 50 mM iodoacetate. Similarly neither NAD nor oxaloacetate, separately or together, affected the inactivation caused by iodoacetate.

The specificity of the iodoacetate reaction was investigated by carrying out the freeze–thaw inactivation reaction using

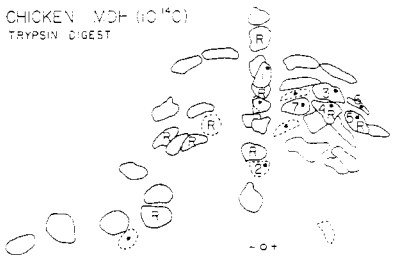

FIGURE 3: Tracing of ninhydrin-stained peptide map pattern of trypsin digest of chicken heart mitochondrial malate dehydrogenase alkylated with 1-[^{14}C]iodoacetate in 8 M urea. Zones labeled "R" gave positive Sakaguchi reaction for arginine. Asterisk (*) indicates radioactive zones determined by autoradiography. Numbered radioactive zones correspond to [^{14}C]carboxymethylated peptides shown in Figure 2E. Zones outlined with dashed lines gave weak or negative ninhydrin color reaction. Electrophoresis: pH 6.4 pyridine acetate for 3 hr at 25 V/cm on Whatman No. 3MM paper. Chromatography: ascending using 1:1 pyridine–isoamyl alcohol saturated with water.

FIGURE 4: Tracing of ninhydrin-stained peptide map pattern of trypsin digest of pig heart mitochondrial malate dehydrogenase alkylated with 1-[^{14}C]iodoacetate in 8 M urea. Symbols and conditions same as in Figure 3 except that numbered radioactive zones correspond to peptides shown in Figure 2A.

1-[^{14}C]iodoacetate. After the freeze–thaw inactivation, the free 1-[^{14}C]iodoacetate was removed by dialysis and the viving thiol groups were blocked by alkylation with unlabeled iodoacetate in 8 M urea. The enzyme had incorporated 4 moles of 1-[^{14}C]carboxymethyl groups per mole. After dialysis against 0.1 M ammonium bicarbonate, the enzyme was digested with trypsin and examined by peptide mapping and autoradiography. The autoradiograph of the peptide map pattern is shown in Figure 2F. It is apparent that 1-[^{14}C]iodoacetate was incorporated into the enzyme at many different sites during the freeze–thaw procedure. Thus, chicken heart mitochondrial malate dehydrogenase does not appear to have various classes of thiol groups reactive to iodoacetate as do the lactate dehydrogenases (Fondy *et al.*, 1965), nor does it possess essential thiol groups specifically available for alkylation by iodoacetate in the native enzyme, or in the enzyme during freezing and thawing.

Carboxymethylation of Denatured Chicken Heart Mitochondrial Malate Dehydrogenases and Peptide Mapping. Because of the lack of specificity of alkylation in the absence of denaturing agents, as described in the previous section, it was decided to totally label all thiol groups in the presence of 8 M urea. Alkylation of three different preparations of the enzyme with a 5:1 molar excess of iodoacetate to thiol groups (70 moles of iodoacetate per mole of enzyme) in 0.1 M Tris-chloride (pH 8.2) and 8 M urea produced an enzyme which had 10 moles of bound [^{14}C]carboxymethyl groups per mole. In our previous work on lactate dehydrogenases these conditions (Fondy *et al.*, 1965) led to incorporation of [^{14}C]carboxymethyl groups almost exclusively into cysteine residues since more than 95% of the radioactivity was localized in [^{14}C]-carboxymethylcysteine. Analysis of the major labeled peptides that were subsequently isolated from the malate dehydrogenases in this present work showed that they contained carboxymethylcysteine residues.

The labeled enzyme preparations were subjected to peptide mapping, followed by ninhydrin staining, autoradiography, and staining for arginine. A tracing of a composite of several peptide maps is shown in Figure 3. With one preparation of the enzyme we found approximately 35 ninhydrin-positive peptides and nine zones which gave a positive test for arginine. The other enzyme preparations gave approximately 30 ninhydrin-positive peptides and a total of 12 arginine-containing zones. Autoradiography showed that there were seven major zones containing [^{14}C]carboxymethyl groups. The autoradiograph is shown in Figure 2E. These studies of total tryptic peptides, arginine peptides, and carboxymethylated peptides are all consistent with the conclusion that the chicken heart mitochondrial malate dehydrogenase preparations used in these studies are composed of 2 subunits which are either identical or very closely similar in primary sequence. From amino acid analysis data (Kitto and Kaplan, 1966), one would expect approximately 35 tryptic peptides, 7-[^{14}C]carboxymethylcysteine peptides, and 10 arginine peptides if the enzyme were composed of two such subunits.

The carboxymethylated peptides were eluted from unstained tryptic peptide maps for further purification and amino acid analysis. These studies will be detailed in a later section.

Carboxymethylation of Denatured Pig Heart Mitochondrial Malate Dehydrogenase and Peptide Mapping. Labeling in 8 M urea and peptide mapping identical with that just detailed for the chicken heart enzyme were carried out on the pig heart mitochondrial enzyme. The ninhydrin-stained pattern is shown in Figure 4 and the autoradiographs of both tryptic and tryptic–chymotryptic digests are shown in Figure 2A,B. The results are very similar to what was found for the chicken enzyme. The pig heart enzyme incorporated 10 g-atoms of ^{14}C per mole of enzyme and showed approximately 30 ninhydrin-positive zones, 9 arginine-containing peptides, and 7-[^{14}C]-carboxymethylated peptides. The amino acid composition of the enzyme (Thorne, 1962) indicates that one would expect 36 tryptic peptides and 8 arginine-containing peptides if the enzyme were composed of two very closely related subunits. These results are consistent with the previous work of Devenyi *et al.* (1966a) on the pig heart mitochondrial enzyme.

The [^{14}C]carboxymethylated peptides from unstained maps of tryptic and tryptic–chymotryptic digests were eluted, purified by paper electrophoresis, and subjected to amino acid analysis. These results will be detailed in a later section.

Carboxymethylation of Denatured Tuna Heart Mitochondrial Malate Dehydrogenase and Peptide Mapping. The results of [^{14}C]iodoacetate labeling in 8 M urea and peptide mapping of a tryptic digest of the tuna heart enzyme are shown in Figure 5. Conditions were the same as used for the chicken and pig heart enzymes. The tuna enzyme incorporated only 6 g-atoms of ^{14}C per mole of enzyme even though amino acid analysis (Kitto and Lewis, 1967) indicates that complete

alkylation of cysteine should give 14 g-atoms of ^{14}C per mole of enzyme. Autoradiography however showed six major radioactive zones. The autoradiographs of both tryptic and tryptic–chymotryptic digests are shown in Figure 2C,D. By amino acid analysis one would expect 7 carboxymethylcysteine-containing peptides if this enzyme were also composed of two very closely related subunits. Thus the autoradiography indicates that the incomplete labeling is probably the result of failure to denature all of the enzyme in 8 M urea, rather than failure to alkylate certain thiol-containing regions in all the enzyme molecules present. Ninhydrin staining showed approximately 25–30 tryptic peptides, noticeably fewer than the number observed in the pig and chicken heart enzymes. The tuna enzyme has a total of 59 lysine and arginine residues which is about 10 fewer than the total present in the pig and chicken enzyme, and is therefore consistent with the reduced number of tryptic peptides in the tuna enzyme.

Comparative Amino Acid Composition of 1-[^{14}C]Carboxymethylated Peptides from Trypsin and from Trypsin–Chymotrypsin Digests of 1-[^{14}C]Carboxymethylated Pig, Chicken, and Tuna Heart Mitochondrial Malate Dehydrogenases. Aliquots of the trypsin digests of malate dehydrogenase preparations shown in Figures 3, 4, and 5 were also subjected to peptide mapping without subsequent ninhydrin staining. Instead, the peptides containing 1-[^{14}C]carboxymethyl groups were localized by autoradiography, cut out from the maps, and eluted with 10% pyridine. The peptides were further purified by electrophoresis at pH 3.5, again eluted from the papers, freeze-dried, and subjected to amino acid analysis after acid hydrolysis. The same procedure was repeated for trypsin–chymotrypsin digests of the alkylated pig and tuna heart enzymes. The results of the analysis of the purified peptides are collected in Figure 2. The amino acid composition of each peptide is listed in Table I. The migration of each peptide at pH 3.5 is given in centimeters per hour with the conditions of the electrophoretic runs being held constant throughout the work. The amount of peptide isolated after two elutions from paper ranged from 5 to 30 mμmoles requiring the use of long path cuvets for amino acid analysis.

It is important to emphasize that the compositions shown in Figure 2 are the result of single analyses done on from 3 to 20 mμmoles of purified peptide hydrolysate per column. Such results should not be considered to be as reliable as analyses done on larger quantities of peptides obtained by preparative column purification methods or by paper methods beginning with larger amounts of digest. In the cases where we did ion-exchange column isolation of micromole amounts of some of the peptides shown in Figure 2, the analysis of the peptides was confirmed qualitatively and quantitatively. Nevertheless, the compositions shown in Figure 2 should be considered as the equivalent of specific staining methods in the qualitative determination of amino acid content, and should not be confused with rigorous quantitative analyses of peptides required for primary sequence determination. This is particularly true of those peptides which failed to migrate during electrophoresis at pH 3.5.

The object of these comparative amino acid composition studies was to determine if a given thiol peptide was retained virtually unchanged during the evolution of malate dehydrogenases, in a manner analogous to that observed for the active-site thiol peptide of lactate dehydrogenases (Fondy *et al.*, 1965; Fondy and Kaplan, 1965; Mella *et al.*, 1968).

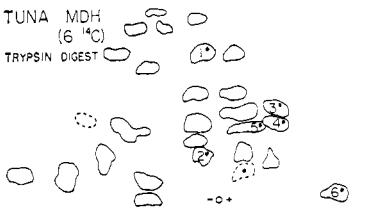

FIGURE 5: Tracing of ninhydrin-stained peptide map pattern of trypsin digest of tuna heart mitochondrial malate dehydrogenase alkylated with 1-[^{14}C]iodoacetate in 8 M urea. Numbered radioactive zones correspond to peptides shown in Figure 2C. Other symbols and conditions of peptide mapping are the same as in Figure 3.

It appears from a study of Figure 2 that the peptides designated "2" in the trypsin digests of the pig and chicken heart enzymes migrate identically in the peptide maps of both species, behave identically during electrophoresis at pH 3.5, and exhibit the same amino acid composition. A similar peptide appears in the trypsin digest of the tuna enzyme but the amino acid analysis of the peptide indicated that it was still impure after electrophoresis at pH 3.5. However, analysis of peptides designated "2" in the trypsin–chymotrypsin digests of the pig and tuna heart enzymes indicated that they were pure and that these trypsin–chymotrypsin peptides from the two species are identical in amino acid composition. Peptide 2 in the trypsin–chymotrypsin digest of the pig enzyme is apparently derived from peptide 2 in the trypsin digest since these peptides differ only in one threonine residue. Peptide 2 in the trypsin digest may also be a chymotryptic fragment of peptide 1. Peptide 2 in the tuna enzyme trypsin–chymotrypsin digest may be derived either from peptide 2 in the trypsin digest or from peptide 1. It would appear that the peptides designated "2" in the various digests of the heart mitochondrial malate dehydrogenases from pig, chicken, and tuna represent a thiol-containing region in these enzymes which is conserved during evolution. Amino-terminal sequence analysis of the peptide from chicken heart mitochondrial malate dehydrogenase to be detailed in the next section showed isoleucine to be the terminal residue. On the other hand, threonine may be at the amino-terminal position in the peptide from pig heart malate dehydrogenase if peptide 2 in the trypsin–chymotrypsin digest is indeed derived from peptide 2 in the trypsin digest. Thus the sequences of peptide 2 in the chicken and pig enzymes are not likely to be completely identical. In any event, this region is a likely candidate from primary sequence analysis and comparison with sequences around essential thiol groups in other NAD-linked dehydrogenases.

The peptides designated "1" in the trypsin digests of the pig, chicken, and tuna enzymes also appear to be similar or identical with one another in the properties shown, although determinations for glycine, alanine, threonine, and serine in some cases as shown in Figure 2 cause some uncertainty. It is possible that peptide 2 in the trypsin–chymotrypsin digests of the pig and tuna enzymes is derived from peptide 1 in the trypsin digests, and that peptide 2 in the trypsin digest of the pig enzyme is really a chymotryptic fragment of peptide 1 in the trypsin digest. Peptide 1 in the trypsin digest of the tuna enzyme may be converted into peptide 2 in the trypsin–chymotrypsin digest. These relationships suggest that the peptides designated "1" in the trypsin digests of the three

TABLE I: Amino Acid Content of [^{14}C]Carboxymethylated Peptides from Digests of Pig, Chicken, and Tuna Heart Mitochondrial Malate Dehydrogenases Shown in Figure 2 after Elution and Electrophoretic Purification at pH 3.5.

	Tryptic peptide																						
	1			2			3			4				5				6			7		
Amino Acid	P[a]	C	T	P	C	T*	P*	C*	T[c]	P	C		T	P	C		T*	P	C[c]	T	P	C*	
											a	b*			a	b*							
Lysine	1	1	1	1	1	1	p[b]	1		1	1	1		1		1				1		p	
Histidine							p			1													
Arginine											1		1		1						1		
CM-cysteine	1	1	1	1	1	1	p	1		1	2	1	1	1	1	1	1	1		1	1	p	
Aspartic						1	p	2		2	1	1				2	2	1			1	p	
Threonine	1	2	p	1	1		p	2		2		1				1	1	2				p	
Serine	2	1	p	1	1	2	p	1		1		1		1	1	1	1	2				p	
Glutamic	1	1	1	1	1	1	p	1		1	3	1	1	2	2	2	2	2		3		p	
Proline	1	2	2	1	1	2	p	1		1	3					1	1	1			2	p	
Glycine	1	p	1			2	p				4	1	1	1	2	1	1				2		
Alanine	1	p	p			2	p	2		2	1	1				1	2				1	p	
Valine						1	p	1		2	3	p	3	2	2	1	2				3	p	
Methionine								1														p	
Isoleucine	2	2	2	1	1	1	p	1		2	1	p			1	1	2				1	p	
Leucine	1	1	1			1	p	1		1	2	p				1	1	1					
Tyrosine						1								1									
Phenylalanine						1	p	1		1	1		1	1	1	1		1				p	
Electrophoretic migration (cm/hr)	10.3	10.3	9.6	13.0	12.3	13.6	0	0		0	9.0	0	9.2	9.6	9.0	0	10.0	3.3		10.8	8.0	0	

	Tryptic–chymotryptic peptide															
	P*		T*	P		T	P	T	P*		T	P			T	P
												a	b*			
Lysine				1		1			1		1	1				
Histidine																
Arginine			1													
CM-cysteine	1		1	1		1		1	1		1	1	1			
Aspartic	1		1						1				1			1
Threonine													1			
Serine				1		1		1	1			1	1			

Glutamic	2		1	1	2	1	3	2	1		2			
Proline	1		1	1					1					
Glycine		1			2	2		1			2			
Alanine		3				1			2		1			
Valine					2	1		2	1		3			
Methionine									1					
Isoleucine			1	1					2		1			
Leucine														
Tyrosine														
Phenylalanine					1			1	1					
Electrophoretic migration (cm/hr)	2.8	11.3	12.3	14.6	2.0	9.0	10.6	8.0	0		8.0			

[a] P, pig; C, chicken; T, tuna; asterisk (*) indicates peptide was still impure after electrophoresis at pH 3.5. [b] p indicates amino acid residue present but could not be reliably quantitated. [c] Not analyzed.

species of mitochondrial malate dehydrogenases are also of particular interest in comparative primary sequence studies.

Correlation of Tryptic Peptides in This Study of Pig Heart Mitochondrial Malate Dehydrogenase with Those Found Previously. The labeling of the pig heart enzyme in this study was done essentially by the same procedure as used by Devenyi *et al.* (1966a) in their work on the pig heart enzyme. They found six different thiol peptides in a tryptic digest and determined the amino acid compositions and some primary sequences. Table II shows the correspondence between the peptides in our work and those of Devenyi *et al.* (1966a,b). Our tryptic peptides "4" and "3" appear to correspond to those designated "T-N_2a" and "T-N_2b" in the earlier work, although our peptide "3" was not sufficiently pure to permit a quantitative analysis. The peptide designated "T-4" by Devenyi *et al.* (1966a,b) corresponds reasonably well to our tryptic peptide "5a," but corresponds better to the peptide designated "5a" in our trypsin–chymotrypsin digest of the pig enzyme.

We did not find any peptide in our maps of the trypsin digest of the pig heart enzyme which corresponded to the peptides designated "T-6a" and "T-6b" by Devenyi *et al.* (1966a,b). Our peptide 1 in the trypsin–chymotrypsin digest, however, did appear to be very similar to those two peptides, particularly to "T-6b." Devenyi *et al.* (1966a,b) listed a total of 4 residues in peptide "T-6b" and 5 in "T-6a," but one amino acid in each was apparently not specified in Table I in that work. It does appear likely however that those peptides are chymotryptic fragments of tryptic peptides and correspond to our tryptic–chymotryptic peptide 1. Thus the absence of basic amino acid in peptides "T-6a" and "T-6b" of Devenyi *et al.* (1966a,b) does not necessarily indicate that these peptides represent the carboxy terminus in the enzyme subunits.

We were unable to find an obvious correspondence between any peptides found in our trypsin or trypsin–chymotrypsin digests and those designated "T-2" and "T-3" by Devenyi *et al.* (1966a,b). Similarly the peptides designated "T-1" and "T-2" in our tryptic peptide maps do not correspond directly to any peptides found by the earlier workers. It is possible that the absence of complete correspondence between the results of these two studies arises from different levels of chymotryptic impurities in the trypsin preparations used in both studies and that comparative trypsin–chymotrypsin peptide maps might have shown a better correspondence. We prepared autoradiographs of peptide maps of the alkylated pig heart enzyme after digestion with a trypsin preparation freed of chymotrypsin by treatment with tosylamidophenylethyl chloromethyl ketone. The autoradiographs were identical with those presented in this work. It is also possible that absence of genetic identity between the populations of pigs used as the enzyme sources in both studies would prevent obtaining complete correspondence in any case.

Preparative Column Fractionation of [^{14}C]Carboxymethylated Peptides from Trypsin Digest of [^{14}C]Carboxymethylated Chicken Heart Mitochondrial Malate Dehydrogenase. Chicken heart mitochondrial malate dehydrogenase (90 mg) was carboxymethylated with 1-[^{14}C]iodoacetate and shown to contain 14 g-atoms of ^{14}C per mole of enzyme. The dialyzed enzyme was digested with trypsin, freeze-dried, dissolved in 0.2 M pyridine acetate pH 3.1, and applied to a 1.2 × 100 cm column of Dowex 50-X2. The column was developed as

TABLE II: Correspondence between Tryptic Peptides Obtained by Devenyi *et al.* (1966a) from [^{14}C]Carboxymethylated Pig Heart Mitochondrial Malate Dehydrogenase and Those Found in This Study.

	Devenyi Peptide		This Work[a]		Devenyi Peptide	This Work[a]		Devenyi Peptide	This Work[a]
Amino Acid	T-N_2a	T-N_2b	T-4	T-3	T-4	TC-5a	T-5a	T-6b	TC-1
Lysine	1	2	1	p[b]	1	1	1		
Histidine	1	1	1	p					
Arginine									
CM-cysteine	1	1	1	p	1	1	1	1	1
Aspartic	2	2	2	p					1
Threonine	2	2	2	p					
Serine	1	1	1	p		1	1		
Glutamic	2	3	1	p	2	2	2	1	2
Proline	2	3	1	p				1	1
Glycine	1	1		p	1	1	1		
Alanine	2	2	2	p					
Valine	1	1	2	p	3	2	2		
Isoleucine	2	3	2	p					
Leucine	1	1	1	p					
Tyrosine							1		
Phenylalanine			1	p	1	1	1		

[a] Letter "T" indicates peptide from tryptic peptide map of pig heart enzyme as shown in Figure 2. Letters "TC" indicate peptide from tryptic–chymotryptic peptide map. [b] p indicates that amino acid is present but impurities prevent reliable quantitative determination.

TABLE III: Amino Acid Compositions of Electrophoretically Purified Dowex 50-X2 Column Fractions of Trypsin Digest of [^{14}C]-Carboxymethylated Chicken Heart Mitochondrial Malate Dehydrogenase, and Comparison with Corresponding Tryptic Peptides from Peptide Mapping (Figure 2).

Amino Acid	Fraction 304	Peptide T-1	Fraction 312	Peptide T-4a	Fraction 328	Peptide T-2	Fraction 356	Peptide T-5a
Lysine	0.9	1	1.0	1	0.7	1		
Histidine								
Arginine			1.0	1			0.8	1
CM-cysteine	0.6	1	1.5	2	0.8	1	0.8	1
Aspartic			0.8	1				
Threonine	1.3	2			0.8	1		
Serine	0.9	1			0.8	1	1.1	1
Glutamic	1.1	1	3.0	3	1.0	1	2.0	2
Proline	3.5	2	4.5	3	0.9	1		
Glycine	2.0	*a*	4.4	4			1.6	2
Alanine	0.7	*a*	1.1	1				
Valine			3.1	3			1.9	2
Isoleucine	1.6	2	0.8	1	0.9	1	0.8	1
Leucine	1.0	1	2.8	2				
Tyrosine								
Phenylalanine			1.1	1			1.0	1
Electrophoretic migration (cm/hr)								
pH 6.4, 44 V/cm	−0.7	−0.4	6	4				
pH 3.5, 50 V/cm					−13	−12	−10	−9

[a] Present, but not reliably quantitated.

TABLE IV: Partial Sequence Analysis of Peptide "328" (Tryptic Peptide 2) from [^{14}C]Carboxymethylated Chicken Heart Mitochondrial Malate Dehydrogenase.

	Lysine	[^{14}C]CM-cysteine	Threonine	Serine	Glutamic	Proline	Isoleucine
Initial peptide	0.7	0.8	0.8	0.8	1.0	0.9	0.9
After first Edman cycle	0.7	0.9	0.8	0.7	1.0	1.0	0.1
After second Edman cycle	*a*	<0.3	0.8	0.7	0.5	*b*	0

[a] Not analyzed. [b] Present, but could not be quantitated.

shown in Figure 1, and the effluent monitored for radioactivity and for ninhydrin color after alkaline hydrolysis (Hirs *et al.*, 1956). Six discrete radioactive zones were identified, collected, and freeze-dried. The fractions were designated according to the tube number containing the peak radioactivity. Since 2-ml fractions were collected, these designations are one-half the effluent volume for the peaks shown in Figure 2, namely: "120," "220," "304," "312," "328," and "356."

The first four fractions were purified by paper electrophoresis at pH 6.4, 4000 V, on 90-cm strips of Whatman No. 3MM paper. Fractions 120 and 220 did not migrate and amino acid analysis showed that they were still impure after electrophoresis. Fraction 304 moved 1 cm/hr toward the negative pole at pH 6.4 and 4000 V, and fraction 312 migrated 6 cm/hr toward the positive pole under the same conditions. The amino acid compositions of these fractions are listed in Table III along with the compositions of their corresponding peptides in the trypsin digest of the chicken heart enzyme shown in Figure 2E. Fraction 304 is seen to correspond with peptide 1 in the chicken heart enzyme tryptic peptide map, and fraction 320 corresponds to peptide 4a.

Fractions 328 and 356 were subjected to electrophoresis at pH 3.5 under the conditions used for the purification of peptides in Figure 2. Fraction 328 moved 13 cm/hr toward the negative pole and thus appeared to correspond to peptide 2 in the tryptic peptide map of the chicken enzyme shown in Figure 2E. Fraction 356 migrated 10 cm/hr toward the negative pole and amino acid analysis (Table III) showed that it corresponded to peptide 5a in the tryptic peptide map in Figure 2E.

Since fraction 328 was thought to correspond to peptide 2 which appeared to be conserved during the evolution of malate dehydrogenases, it was subjected to further purification prior to analysis. This purification was carried out on a 0.9 × 52 cm column of Dowex 1-X2 as detailed under Methods. The peptide was eluted in a sharp zone with a peak at 40 ml after the start of the gradient. The pooled fractions containing the purified peptide were freeze-dried; the peptide was examined for amino acid composition, and subjected to partial sequence analysis. The composition of the peptide confirmed that it was indeed peptide 2 from the tryptic peptide map (Table III). The results of sequence analysis are presented in Table IV. A tentative partial sequence can be assigned from these results assuming that lysine is carboxy terminal in this tryptic peptide. The likely partial sequence is: Ile-CMCys-(Thr,Ser,Pro,Glx)-Lys. The assignment of carboxymethylcysteine to the second position is based on the loss of radioactivity in the peptide after the second Edman cycle and on substantial reduction of that amino acid in the analysis of the residual peptide as shown in Table IV. However, glutamic acid also diminished in that cycle and the small amount of peptide remaining did not permit a reliable proline determination, making the positioning of carboxymethylcysteine rather uncertain.

Discussion

It has been generally observed in the study of protein evolution that the functionally essential regions in the primary sequences of proteins are more rigidly conserved than the rest of the sequence. This generalization is true for lactate dehydrogenases and glyceraldehyde-3-P dehydrogenases as cited in an earlier section. It is true most notably for the cytochromes *c* (Nolan and Margoliash, 1968), and for the serine proteases (Neurath *et al.*, 1967; Smillie and Hartley, 1966). Indeed, the remarkable conservation of the amino acid sequence from positions 70 to 80 in cytochromes *c* had been the subject of considerable speculation even before the essential function of the methionine residue at position 80 in heme binding was demonstrated (Margoliash and Smith, 1965; Nolan and Margoliash, 1968).

If a particular type of amino acid residue is known from chemical or physical studies to have an essential role in the operation of a protein, it should not be difficult to determine exactly which residue is the essential one from a comparison of complete primary sequences of the protein from two or several quite diverse species. This type of comparative identification of an essential residue is useful only in a few cases however, since covalent labeling studies usually precede total sequence analysis. The question addressed in our work is: "Can one get an indication of the portion of a protein sequence containing a functionally essential residue, in the absence of comparative sequence information, even if the essential residue has resisted attempts at covalent labeling? Further, can such an indication of a functionally essential region by a comparative approach be obtained by methods that are experimentally practical?"

From our work on the mitochondrial malate dehydrogenases of pig, chicken, and tuna heart we have been able to identify a specific peptide containing a modified thiol residue, which appears in proteolytic digests of each of the three malate dehydrogenases. This peptide appears to be quite similar or identical in electrophoretic and chromatographic

properties, and in amino acid composition in all three enzymes. We have also learned that it is useful to work with both trypsin and trypsin–chymotrypsin digests, since the latter are likely to give thiol peptides with fewer total residues, thus simplifying the identification of the thiol peptide common to all three species of the enzyme.

Peptide mapping is particularly useful for comparative identification of essential regions because it permits the ready comparison among several different protein digests of the peptides containing the type of residue in question. The chief shortcoming of the peptide mapping technique is the low yield of peptide obtained after elution and purification, which makes confirmatory primary sequence determination difficult or impossible.

While we have identified a particular thiol peptide in mitochondrial malate dehydrogenases which appears to be conserved during evolution, it is difficult to evaluate the uniqueness of this conservation. It is apparent that not all six or seven thiol peptides in the malate dehydrogenases studied have been conserved. However, lack of sufficient material precluded complete characterization of all thiol peptides, particularly the larger peptides, where amino acid analysis alone was insufficient to indicate whether these peptides are conserved or altered during evolution. Thus the peptide mapping approach used in this work can indicate a thiol peptide which appears to be conserved in the enzymes studied, but cannot establish whether some other thiol peptide might also be similarly conserved.

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