THE PRIMARY STRUCTURE OF THE LACTATE DEHYDROGENASE ISOZYME M_4 FROM GIANT PANDA

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ABSTRACT

The amino acid sequences of tryptic, chymotryptic and cyanogen bromide cleavage peptides of the lactate dehydrogenase isozyme M_4 from giant panda have been determined. Based on the overlapping peptides and by a comparison with the known sequence of porcine lactate dehydrogenase isozyme M subunit, the complete primary structure of the giant panda lactate dehydrogenase isozyme M subunit has been established. The polypeptide chain of giant panda lactate dehydrogenase isozyme M subunit consists of 331 amino acid residues. There is a variance of 17 amino acid residues between the porcine and the giant panda lactate dehydrogenase isozyme M subunits. Most of the variable residues are substitutions by chemically similar amino acids and take place at residues not related to the active center of the enzyme.

INTRODUCTION

The evolution and the taxonomic position of the giant panda (*Ailuropoda melanoleuca*), a rare animal indigenous to China, has long been a controversial question. Some scientists such as Bardenfleth[1] and Davis[2], based on their comparative anatomical observations on the giant panda, concluded that the giant panda is just an aberrant bear and therefore is a belonging to the Ursidae. Others like Mivart[3] and Raven[4] considered it is closely related to the red panda, *Ailurus fulgens*, and both are belongings to the Procyonidae. Still others like Pocock[5] and Zhu[6], suggested that the giant panda should be classified as an independent family— the Ailuropodidae.

In recent years scientists have resorted to biochemical methods to provide information on questions of evolution and taxonomic position of the giant panda. The comparative study of the primary structures of the protein cytochrome c of various organisms has led to the belief that the amino acid sequence of a protein can be very helpful as a taxonomic feature. The first attempt to use protein sequence to study the phylogenetic relationship of the giant panda was the work of Borden and Margoliash[7] on the cytochrome c of the giant panda. By comparing the peptide maps of chymotryptic and tryptic digests of giant panda and dog cytochromes c prepared by thin layer two-dimensional electrophoresis-chromatography they found that the fingerprints of the peptide fragments are virtually identical and thus they concluded that the amino acid sequences of dog and giant panda are identical. Furthermore, they inferred the primary structures of cytochromes c are likely to be of little value when comparisons of these proteins are made for closely related species because of the paucity of variations.

Abbreviations: LDH, lactate dehydrogenase; TPCK, N-tosyl-L-phenylalanyl-chloromethyl ketone; DABITC, 4-N,N N-dimethylaminobenzene-4′-isothiocyanate.

It is known that the rates of change of protein sequences in evolution are different for different proteins. By choosing the proper protein and by comparison of the primary structures of these homologous proteins between the giant panda and other related mammals it might be possible to obtain instructive phylogenetic information. This is the rationale which initiated the present investigation.

Among the proteins which could be isolated from various tissues of the giant panda, we have chosen the lactate dehydrogenase (EC. 1. 1. 1. 27) isozyme M_4 as our object because it is an "ancient" protein which plays an important role in metabolic activities. This enzyme emerged ever since anaerobic fermentation became a metabolic pathway in the life of organisms. It appears to be associated with all known species. Its isozyme M_4 occurs in abundant amount in muscles of animals and can be easily purified by affinity chromatography. Lactate dehydrogenase isozyme M subunit has a molecular weight 2.7 times that of cytochrome c and its rate of change of protein sequence in evolution is faster than that of cytochrome c. These factors make it possible for more variations to occur in protein sequence of this enzyme protein accumulated during the long process of evolution.

The purification and partial characterization of lactate dehydrogenase isozyme M_4 from giant panda and the analysis of the HPLC tryptic peptide map of this enzyme have been reported by this laboratory[8,9]. The present investigation deals with the determination of the complete primary structure of giant panda lactate dehydrogenase isozyme M_4.

I. Materials and Methods

1. *Materials*

Crystalline giant panda LDH-M_4 and porcine LDH-M_4 were prepared according to the method described in a previous paper[8]. TPCK-Trypsin and chymotrypsin were from Sigma. Cyanogen bromide was from Merck. Sephacryl S-200 and Sephadex G-75 were products of Pharmacia. Acetonitrile was obtained from Huangyan Chemicals, HPLC grade. All other reagents were of A. R. grade.

2. *Methods*

The peptide fragments obtained from the trypic digests of giant panda and porcine LDH-M_4 were isolated by reverse-phase HPLC according to the previously published procedure[8,9].

Conditions for the hydrolysis of giant panda carboxymethylated LDH-M_4 by chymotrypsin were essentially the same as those by TPCK-trypsin. 2 mg of carboxymethylated LDH-M_4 were dissolved in 1 ml of distilled water and 1 ml of 0.2 *M* N-methylmorpholine solution, pH 8.0, was added. After the addition of 40 μl of chymotrypsin solution (1 mg/ml), the mixture was incubated at 37°C for 2 h. The digest was heated in a boiling water bath for 3 min and then lyophilized.

The procedure of Steers[10] was used for cyanogen bromide cleavage of giant panda LDH-M_4. 30 mg of LDH-M_4 were dissolved in 6 ml 70% formic acid solution and 100 mg of crystalline CNBr were added. After filling the vessel with nitrogen gas, the reaction mixture was allowed to stand at room temperature in the dark for 24 h. When the reaction was complete, the sample was diluted with 10 volumes of double-distilled water and then lyophilized. The lyo-

philized sample was dissolved in 5 ml of 50% acetic acid solution and then applied to a Sephacryl S-200 column for chromatography. The column was previously equilibrated with 50% acetic acid solution and after the application of the sample, it was eluted with 50% acetic acid solution at a flow rate of 30 ml/h. The fractions collected were lyophilized separately.

When necessary, some of the peptide fractions from chymotryptic digest or CNBr cleavage could be further separated and purified by HPLC under appropriate conditions.

Sequence determination by Edman degradation of the peptides was performed on a Model 470 A Gas-phase Protein Sequencer of Applied Biosystems.

II. Experimental Results and Discussion

1. *Sequence Determination of Peptides From Tryptic Digest of Giant Panda LDH-M_4*

The isolation of peptides for sequence studies from the tryptic digest of giant panda LDH-M_4 and carboxymethylated LDH-M_4 and peptide maps obtained from HPLC were described in a previous paper[9]. All peptides derived from tryptic digests were sequenced, the results of sequence determinations are shown in Table 1. Peptides T2, T3, T34, T36, T37 and the first 13 residues of T29 in Table 1 were also sequenced by manual Edman degradation with the colored reagent DABITC to confirm the results of automated Edman degradation on the Gas-phase Protein Sequencer, and the results were identical. It was expected that the tryptic digest of giant panda LDH-M_4 should give rise to 37 peptide fragments and a free Arg and a free Lys each. Due to the fact that T6 (residues 57 and 58), T28 (residues 243 and 244) and T35 (residues 315 and 316) were of the same amino acid composition and of the same sequence Leu-Lys, only 35 different peptide fragments were isolated. In Table 1, the N-terminus of T1 was blocked and no information on Edman degradation could be obtained. Since it had the same amino acid composition, the same retention time and position in HPLC peptide map as T1 derived from the tryptic digest of porcine LDH-M_4 whose primary structure had been determined and was known to have a blocked acetylated Ala as the N-terminus, it could be inferred that both T1 peptides were of the same sequence. Of all peptide fragments derived from tryptic digest of giant panda LDH-M_4, only the sequence of peptide T4 was not available since it did not appear in the HPLC peptide map. It might be possible that this peptide was left in the insoluble portion of the tryptic digest.

2. *Sequence Determination of Peptides From Chymotryptic Digest of Giant Panda LDH-M_4*

With the sequence information derived from the initial hydrolysis of giant panda LDH-M_4 by trypsin, a second cleavage procedure by chymotrypsin with different specificity was used to generate peptide fragments whose sequences extend across the initial cleavage points (overlapping peptides) in order to determine the complete amino acid sequence of the enzyme. Giant panda carboxymethylated LDH-M_4 was digested with chymotrypsin and the resulting peptides were isolated by reverse-phase HPLC. Fig. 1 shows the HPLC peptide map from chymotrypsin digestion. Some of the peptide fragments which were not pure enough for sequence determination were subjected to a second time HPLC under a different set of conditions.

The amino acid composition of chymotryptic peptides is shown in Table 2. All peptides in Table 2 were sequenced by Edman degradation on an Applied Biosystems Model 470 A Protein Sequencer and the results are given in Table 3. Of the total 331 amino acid residues, about 254 residues, or 77%, were sequenced. It can be seen that most of the peptides derived

Table 1

Amino Acid Sequences of Peptides
From Tryptic Digest of Giant Panda LDH-M_4

Peptide	Retention Time (min)	Sequence	Position
T2	61.650	DQLIQNLLK	5—13
T3	14.447	EEHTPQNK	14—21
T5	56.697	DLADELALVDVIEDK	42—56
T6	6.903	LK	57—58
T7	73.164	GEMMDLQHGSLFLR	59—72
T6+T7	75.158	LKGEMMDLQHGSLFLR	57—72
T8	5.606	TPK	73—75
T9	22.582	IVSGK	76—80
T10	28.159	DYNVTANSK	81—89
T11	68.025	LVI ITAGAR	90—98
T12	28.159	QQEGESR	99—105
T13	52.724	LNLVQR	106—111
T14	58.988	NVNIFK	112—117
T15	88.910	FI IPNIVK	118—125
T16	37.069	YSPNCK	126—131
T17	104.477	LLVVSNPVDILTYVAWK	132—148
T18	50.788	ISGFPK	149—154
T19	16.728	NR	155—156
T20	45.874	VIGSGCNLDSAR	157—168
T21	19.637	FR	169—170
T22	43.872	YLMGER	171—176
T23	87.289	LGVHPLSCHGWVLGEHGDSSVPVWSGVNVAGVSLK	177—211
T24+T25	44.286	NLHPDLGTDADKEQWK	212—227
T26	12.741	QVHK	228—231
T27	48.711	QVVDSAYEVIK	232—242
T29	91.491	GYTSWAIGLSVADLAESMMK	245—264
T30	19.637	NLR	265—267
T31	69.535	RVHPISTMIK	268—277
T31	66.349	VHPISTMIK	267—277
T32	55.446	GLYGIK	278—283
T33	77.423	DDVFLSVPCILGQNGISDVVK	284—304
T34	31.834	VTLTPEEEAR	305—314
T36	52.724	SADTLWEIQK	318—327
T37	37.069	ELQF	328—331
T36+T37	75.610	SADTLWEIQKELQF	318—331

from the second cleavage procedure by chymotrypsin contained overlapping peptides which were informative in ordering and lining up the initial set of tryptic peptides. Sequence determination of chymotryptic peptides was not only significant for the establishment of the primary structure of giant panda LDH-M subunit, furthermore, it also confirmed the accuracy of sequence determination of the peptide fragments derived from the initial cleavage by trypsin.

Among the chymotryptic peptides there were 77 residues whose sequences were not determined. Most of these residues were near the N-terminus of the polypeptide chain. It is to be pointed out that the aforementioned T4 peptide from tryptic digest of giant panda LDH-M_4 was also

Table 3

Amino Acid Sequence of Chymotryptic Peptides From Giant Panda LDH-M_4

Peptide	Retention Time in HPLC Map (min)	Sequence	Position
C1	85.031	MKDLADELALVDVIEDKL	40—57
C2	86.042	ALVDVIEDKLKGEM	48—61
C3	64.221	LRTPKIVSGKDY	71—82
C4	35.254	NVTANSKL	83—90
C5	63.321	VI ITAGARQQEGESRL	91—106
C6	72.391	NLVQRNVNIF	107—116
C7	97.774	KFI IPNIVKY	117—126
C8	78.317	I IPNIVKY	119—126
C9	75.444	LVVSNPVDILTY	133—144
C10	37.492	VAWK	145—148
C11	73.741	KISGFPKNRVIGSGCNLDSARF	148—169
C12	45.431	LMGERLGVHPL	172—182
C13	55.495	VLGEHGDSSVPVW	188—200
C14	67.530	SGVNVAGVSL	201—210
C15	46.940	KNLHPDLGTDADKEQW	211—226
C16	40.495	KQVVDSAY	231—238
C17	49.029	KGYTSWAIGL	244—253
C18	80.292	AIGLSVADLAESM	250—262
C19	57.048	SVADLAESMM	254—263
C20	58.654	RRVHPISTM	267—275
C21	41.520	GIKDDVF	281—287
C22	89.784	LSVPCILGQNGISDVVKVTLTPEEEARL	288—315
C23	54.691	KKSADTLW	316—323
C24	34.025	EIQKELQF	324—331

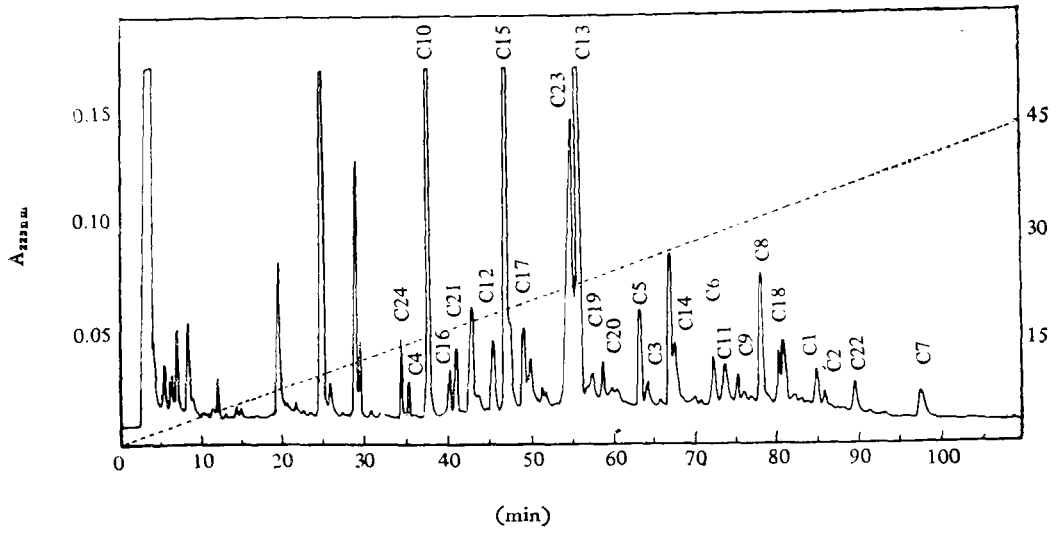

Fig. 1. The separation by HPLC of the chymotryptic peptides of carboxymethylated LDH-M_4 from giant panda.

Column: YWG-C18, 5μm, 4×300mm; temperature: 45℃; flow rate: 0.8ml/min; attenuation: 128; mobile phase: solvent A, 0.01*M* ammonium acetate buffer, pH 5.8, solvent B, 100% ACN.

Table 2

Amino Acid Composition of Chymotryptic Peptides From Giant Panda LDH-M_4

Peptide	Amino Acid Composition																	Sum
	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	Lys	His	Arg	Trp	
C1	4.3			1.8			2.0		1.6	0.8	0.7	4.2			2.2				18
C2	2.1			1.8		1.1	1.0		1.8	0.8	0.9	2.2			2.3				14
C3	0.9	0.9	0.7		0.8	1.3			0.8		0.7	1.2	0.8		2.1		1.0		12
C4	2.1	1.1	0.7				1.0		0.9			1.3			1.0				8
C5		0.7	0.9	4.2		2.2	2.0		0.8		1.7	0.9					1.8		16
C6	2.6			1.1					1.8		0.9	1.1		0.8			1.0		10
C7	1.2				1.1				0.9		2.8		0.8	0.7	2.0				10
C8	1.0				0.9				0.9		3.1		0.7		1.0				8
C9	1.9	0.9	0.8		0.6				3.2		0.8	2.2	0.8						12
C10							1.0		1.0						1.1			0.7	4
C11	2.8		2.6		0.8	3.0	1.0	0.6	0.7		1.7	0.9		1.6	2.2		1.9		27
C12				0.9	0.8	1.9			0.9	0.9		3.1				0.9	1.0		11
C13	0.9		1.7	1.2	0.7	1.8			2.6			1.1				0.8		0.6	13
C14	1.1		1.8			2.1	1.0		2.7			1.2							10
C15	4.2	0.7		1.8	0.8	1.1	1.0					1.8			1.8	1.1		0.8	16
C16	0.8		0.9				1.0		1.7				0.9		1.1				7
C17		0.9	0.8			1.8	1.0				1.1	1.2	0.7		0.9			0.8	10
C18	1.0		1.6	1.1		0.9	2.9		0.8	0.8	0.8	1.9							13
C19	1.2		1.7	0.8			2.0		0.8	1.8		0.9							10
C20		1.1	1.0		0.8				1.1	0.9	1.1					1.0	2.1		9
C21	1.7					0.9			0.8		1.2			0.7	1.0				7
C22	1.0	1.8	1.6	4.3	1.8	2.1	1.0	0.7	3.6		1.9	4.2			1.1		1.8		28
C23	1.1	0.9	0.7				1.0					1.1			2.2			0.8	8
C24				4.2							0.8	1.0		0.8	0.9				8

Notes: 1. Trp was determined after hydrolysis with methanesulfonic acid.
2. Cys was determined after performic acid oxidation.

located in that portion of the polypeptide chain.

3. *Isolation and Sequence Determination of Peptides From CNBr Cleavage of Giant Panda LDH-M_4*

Data from sequence determinations of tryptic and chymotryptic peptides still needed supplementary information for the assignment of the complete amino acid sequence of giant panda LDH-M_4. In view of the presence of several Met residues near the N-terminus of the primary structure of porcine LDH-M subunit, giant panda LDH-M_4 was subjected to CNBr cleavage. The peptide fragments after CNBr cleavage were separated by gel filtration on Sephacryl S-200 column. According to the amino acid composition of giant panda LDH-M_4 and the sequence data of its tryptic peptides, CNBr cleavage should give rise to 7 peptide fragments and 2 Hse (homoserine) residues derived from Met. The result of chromatographic separation is shown in Fig. 2. It can be seen from Fig. 2 that there were 5 absorption peaks when the eluents were monitored at 280 nm. Peaks B4, B5 and B7 were rechromatographed once more on Sephacryl S-200. Peak B1 was further purified by gel-filtration Sephadex G-50, and the absorption of the eluents was monitored at 225 nm. B3 and the mixture of B2 and B6 were further

Table 4

Amino Acid Composition of CNBr Peptides From Giant Panda LDH-M_4

Amino Acids	Peptide						
	B1	B2	B3	B4	B5	B6	B7
Asp	3.4(3)		2.3(2)	13.6(14)	8.3(8)	2.1(2)	5.1(5)
Thr	2.2(3)			3.6(4)	1.5(2)	0.9(1)	2.6(3)
Ser		0.8(1)		8.1(9)	8.1(9)	0.9(1)	2.7(3)
Hse	0.5(1)	0.6(1)	0.5(1)	0.5(1)	0.5(1)	0.5(1)	
Glu	4.5(5)		4.8(5)	5.6(6)	8.5(8)		8.3(8)
Pro	1.1(1)			4.5(5)	2.7(3)	1.0(1)	1.7(2)
Gly	3.5(3)			7.2(7)	10.5(10)		4.2(4)
Ala	2.0(2)	2.0(2)	2.0(2)	5.0(5)	6.2(6)		2.0(2)
Val	3.4(4)		2.4(3)	9.1(11)	8.9(11)	0.8(1)	4.1(5)
Ile	1.4(2)	1.8(2)	1.8(2)	7.9(10)	1.6(2)	0.9(1)	4.6(5)
Leu	3.8(4)	1.1(1)	3.4(3)	10.8(11)	9.6(10)		7.1(7)
Tyr				3.21(4)	1.6(2)		0.8(1)
Phe				4.5(5)			1.7(2)
Lys	3.2(3)		3.1(3)	7.8(8)	6.4(6)	1.0(1)	6.3(6)
His	1.1(1)			1.1(1)	4.8(5)	0.9(1)	
Arg				6.5(7)	1.0(1)	1.9(2)	1.0(1)
Sum	32	7	21	108	84	12	54

Note: Trp and Cys were not determined.

Table 5

Amino Acid Sequences of CNBr Cleavage Peptides From Giant Panda LDH-M_4

Peptide	Sequence	Position
B2	ACAISILM	33—40
B3	KDLADELALVDVIEDKLKGEM	41—61
B4	DLQHGSLFLRTPKIVSGKDYNVTANSKLVIITAGA-	63—97
B5	GERLGVHPLSCHGWVLGEHGDSSVPVWS-	174—201
B6	KNLRRVHPISTM	264—275
B7	IKGLYGIKDDVFLSVPCILGQNGISDVVKV-	276—305

purified by HPLC and the eluents were monitored at 225 nm. This last step gave clear separation of B2 and B6. The amino acid composition of peptide fragments from CNBr cleavage is given in Table 4.

All peptide fragments dedived from CNBr cleavage of giant panda LDH-M_4 were sequenced on a Model 470 A Protein Sequencer and the results are given in Table 5. For the longer peptide fragments like B4, B5 and B7, automated Edman degradation cycles were repeated until indecisive results were obtained.

Peptide fragment B1 gave no PTH amino acid upon Edman degradation, its N-terminus was apparently blocked. The sequence of peptide fragment B2 was identical with the sequence of residues 33—40 of porcine LDH-M subunit whose primary structure was known. This part corresponded to the last part of peptide T4 from the tryptic digest of giant panda LDH-M_4. Since peptides T1, T2 and T3 had been isolated from the tryptic digest of giant panda LDH-M_4 and their amino acid compositions had been determined, by subtracting the total amino acid

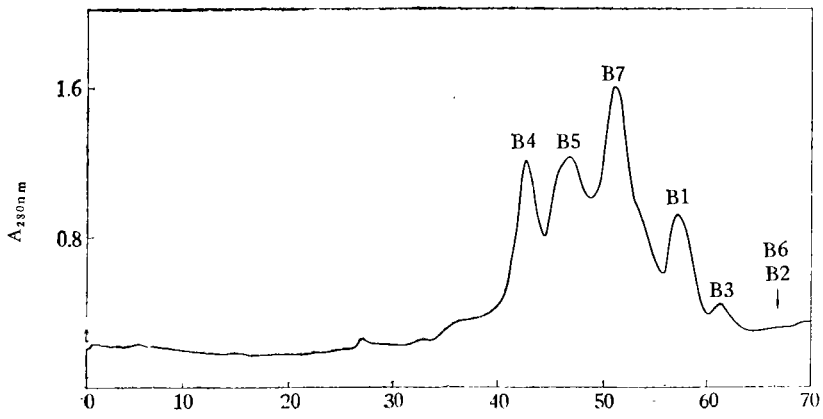

Fig. 2. The separation of CNBr cleavage fragments of LDH-M_4 from giant panda by gel filtration chromatography.
Column: Sephacryl S-200, 2.1×170 cm; flow rate: 30 ml/h; elution solvent: 50% acetic acid; fraction collected: 250 drops/tube.

residues of T1, T2 and T3 from the amino acid composition of peptide B1, the amino acid composition of the first part of peptide T4 could be deduced as follows: Hse (1), Val (4), Gly (3), Thr (1), Ile (1), Ala (1). The residue homoserine evidently was derived from Met. The amino acid composition of the first part of peptide T4 was identical with that of residues 22—32 from porcine LDH-M subunit, their sequences were probably the same.

4. *Preliminary Determination of the Complete Primary Structure of Giant Panda LDH-M Subunit*

The peptide fragments from tryptic, chymotryptic digests and CNBr cleavage of giant panda LDH-M_4 were aligned relative to one another from the information provided by the overlapping peptides. The primary structure of giant panda LDH-M subunit is shown in Fig. 3. Among the complete sequence of the subunit, only residues 22—32 were not directly sequenced. For residues 1—4, no Edman degradation data were available. From the HPLC peptide map of tryptic digest of giant panda LDH-M_4, its amino acid composition and the negative result of N-terminal determination, it could be inferred that this fragment had the same sequence as peptide T1 from porcine LDH-M subunit. No overlapping peptides were available for ordering fragments T1 and T2, T2 and T3, T3 and T4, T25 and T26, and T27 and T28. Their ordering and alignment were inferred by taking into consideration HPLC peptide maps of giant panda LDH-M_4 and porcine LDH-M_4 and from the known sequence of the corresponding fragments of porcine LDH-M subunit.

A comparison of the complete primary structures of giant panda and porcine LDH-M subunits[11] is given in Fig. 4. It can be seen from Fig. 4 that there is a variance of 18 amino acid residues between the primary structures of LDH-M subunits of these two animals (as indicated in Fig. 4 in dotted blocks). It should be noted that in the present investigation, residue 232 of porcine LDH-M subunit was found to be Gln instead of Glu as reported by Kiltz et al.[11] In this case, there is a variance of 17 amino acid residues. Among the 17 variable residues, 9 are replacements by chemically similar amino acids, for example, between Ile, Leu and

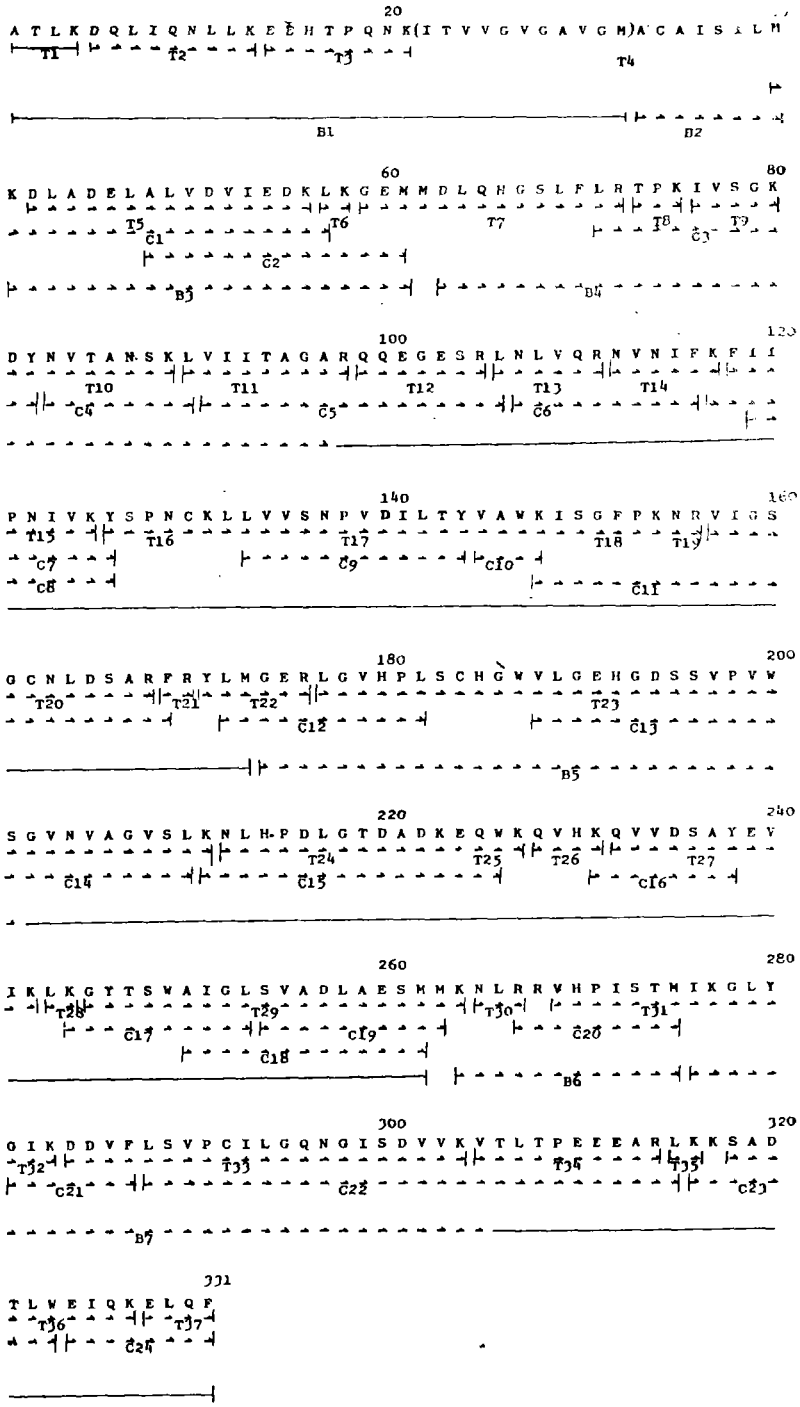

Fig. 3. Summary proof of the primary structure of giant panda LDH-M subunit.

The complete amino acid sequence is presented using the one-letter code. Sequence determined by either manual Edman degradation or by automated protein sequencer is marked by an arrow. The respective peptide fragments obtained by tryptic, chymotryptic and CNBr cleavage are designated by T1, T2,···, C1, C2,···, and B1, B2,···, etc.

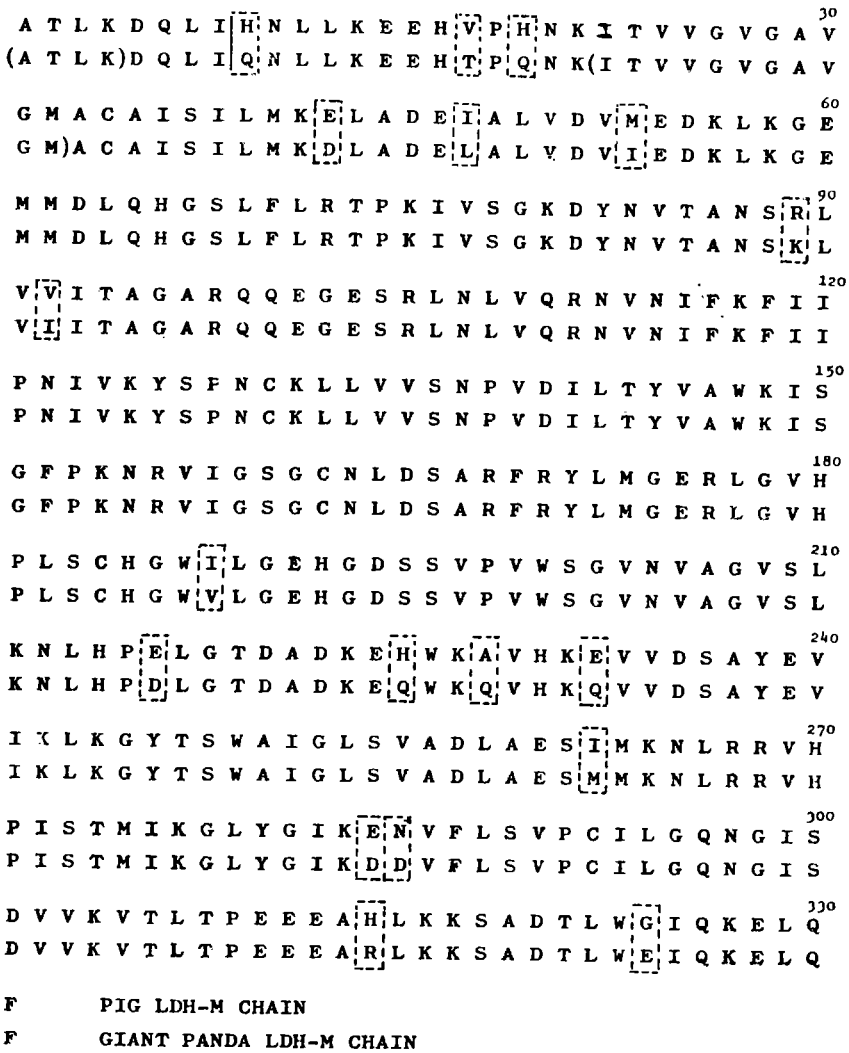

Fig.4. Comparison of amino acid sequences of giant panda LDH-M and pig LDH-M subunits.
Non-identical residues are indicated in dotted blocks.

Val, between Arg and Lys, and between Glu and Asp. Eight are replacements by amino acids of different categories, for example, between His and Gln, between Val and Thr, and between Gly and Glu.

The amino acid residues that are involved in the active center of porcine LDH-M_4 have been described previously. Eventoff et al. have determined the three-dimensional structures of dogfish LDH-M_4 and porcine LDH-H_4 by X-ray crystallographic analysis and correlated their enzyme activity with the amino acid sequences of dogfish LDH-M_4, porcine LDH-M_4, and LDH-H_4, and chicken LDH-M_4 and LDH-H_4[12]. A comparison of active center residues of giant panda LDH-M_4 and porcine LDH-M_4 is given in Table 6. It can be seen from Table 6 that almost all residues involved in the active center of the enzyme are conserved. This means that of the 17 variable amino acid residues between giant panda LDH-M subunit and porcine

Table 6

Comparison of Active Center Residues of Giant Panda LDH-M_4 and Pig LDH-M_4

Site	Number	Pig LDH-M_4	Giant Panda LDH-M_4
Adenine binding	25	V	V
	50	V	V
	51	D	D
	52	V	V
	53	M	I
	82	Y	Y
	93	I	I
	95	A	A
	115	I	I
	120	I	I
Adenine-ribose binding	26	G	G
	28	G	G
	51	D	D
	56	K	K
Pyrophosphate binding	29	A	A
	56	K	K
	96	G	G
	98	R	R
	245	Y	Y
Nicotinamide-ribose binding	30	V	V
	94	T	T
	97	A	A
	136	S	S
Nicotinamide binding	30	V	V
	135	V	V
	137	N	N
	164	L	L
	246	T	T
	251	I	I
Substrate binding	105	R	R
	170	R	R
	192	H	H
Negative ring formation	103	E	E
	104	S	S
	194	G	G
	195	D	D
	232	E	E
	235	D	D
	236	S	S
	238	Y	Y
	239	E	E
Loop arrangement	99	Q	Q

LDH-M subunit, 16 replacements take place at residues not related to the active center of the enzyme. The only one residue, Met 53 of porcine LDH-M subunit which is involved in the binding of the adenine group of the coenzyme NAD, is replaced by Ile in giant panda LDH-M subunit. What is the consequence of this replacement on the association and dissociation between the enzyme and coenzyme remains to be investigated.

The pig and the giant panda both belong to the Class Mammalia whereas the former is a belonging to the Order Artiodactyla and the latter is a belonging to the Order Carnivora. The divergence of Artiodactyla and Carnivora took place at early Palaeocene period, approximately 75 million years ago. It is pointed out that there is a variance of 17 amino acid residues between giant panda and porcine LDH-M subunits and if LDH-M_4 should have a constant rate of change during the long process of evolution as cytochromes c did, then the LDH-M subunits of pig and giant panda both changed 8.5 residues after they branched off from their common ancester. From these data, it can be calculated that the average rate of evolutionary change of LDH-M subunit is 1 amino acid residue in every 8.8 millions of years. This is a much faster rate of change than that of cytochrome c, which changes 1 amino acid residue every 26.4 millions of years. It therefore appears appropriate to choose the lactate dehydrogenase isozyme M_4 as the protein to study the evolution and the taxonomic position of the giant panda.

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