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The Complete Amino Acid Sequences of Cytosolic and Mitochondrial Aspartate Aminotransferases from Horse Heart, and Inferences on Evolution of the Isoenzymes

Shawn Doonan,[1] Filippo Martini,[2] Sebastiana Angelaccio,[2] Stefano Pascarella,[2] Donatella Barra,[2] and Francesco Bossa[2]

[1] Department of Biochemistry, University College, Cork, Ireland
[2] Dipartimento di Scienze Biochimiche e Centro di Biologia Molecolare del CNR, Università di Roma, Città Universitaria, 00185 Rome, Italy

Summary. We report here the complete amino acid sequences of the cytosolic and mitochondrial aspartate aminotransferases from horse heart. The two sequences can be aligned so that 48.1% of the amino acid residues are identical. The sequences have been compared with those of the cytosolic isoenzymes from pig and chicken, the mitochondrial isoenzymes from pig, chicken, rat, and human, and the enzyme from *Escherichia coli*. The results suggest that the mammalian cytosolic and mitochondrial isoenzymes have evolved at equal and constant rates whereas the isoenzymes from chicken may have evolved somewhat more slowly. Based on the rate of evolution of the mammalian isoenzymes, the gene-duplication event that gave rise to cytosolic and mitochondrial aspartate aminotransferases is estimated to have occurred at least 10^9 years ago. The cytosolic and mitochondrial isoenzymes are equally related to the enzyme from *E. coli*; the prokaryotic and eukaryotic enzymes diverged from one another at least 1.3×10^9 years ago.

Key words: Aspartate aminotransferase — Isoenzyme — Protein evolution — Mitochondria

Offprint requests to: S. Doonan

Introduction

The cytosolic and mitochondrial isoenzymes of aspartate aminotransferase (EC 2.6.1.1) are coded for by different, but structurally related, genes. This was first shown conclusively by comparisons of the amino acid sequences of the two isoenzymes from pig heart (Barra et al. 1980)—indeed this was the first pair of cytosolic and mitochondrial isoenzymes for which complete amino acid sequences were established.

Aspartate aminotransferases have been the objects of extensive structural studies in recent years. This interest stems at least in part from the fact that the mitochondrial isoenzyme is synthesized in the cell cytosol and then translocated through the mitochondrial membrane system into the matrix; translocation is a posttranslational event (for a review of these processes, see Doonan et al. 1984a). It seems logical to postulate that the requirement for translocation through the mitochondrial membrane system has imposed extra constraints on the structure of the mitochondrial isoenzyme and that these would be manifest in a slower rate of evolution for this form than for the cytosolic isoenzyme. Comparative studies of aspartate aminotransferases using immunochemical methods suggested that this was indeed the case (Sonderegger et al. 1977; Porter et al. 1981a), and it was claimed that the mam-

malian cytosolic isoenzymes have evolved at more than twice the rate of their mitochondrial counterparts (Sonderegger and Christen 1978). More recent comparisons based on amino acid sequence analysis suggest, however, that there has been little if any difference in rate of evolution between the two isoenzymes (for a review see Doonan et al. 1984b). The sequences on which this conclusion was based were, in addition to those from pig heart, the mitochondrial isoenzymes from chicken (Graff-Hausner et al. 1983) and rat (Huynh et al. 1980) and the cytosolic isoenzyme from chicken (Shlyapnikov et al. 1979); incomplete sequences were also available for the two isoenzymes from horse heart (Martini et al. 1983, 1984).

It is clear that the evolutionary history of aspartate aminotransferases will be placed on a more secure basis as further complete amino acid sequences become available. For this reason we performed sequence analysis of the two isoenzymes from horse heart; we present the results here. The sequence of the cytosolic isoenzyme is of particular interest since it allows for the first time a direct comparison between the structures of this isoenzyme in two mammalian species. The completion of the sequence analysis of the mitochondrial isoenzyme, together with that of the isotopic isoenzyme from human heart (Martini et al. 1985), brings the total of such complete sequences from mammals to four and allows an initial estimate to be made of the rate of evolution of the isoenzyme. The inferences that can be drawn from comparison of all the known sequences are discussed in detail below.

Materials and Methods

The two isoenzymes were isolated from horse heart muscle essentially using the procedure developed in our laboratories for pig-heart aspartate aminotransferases and for the isoenzymes from other sources (Barra et al. 1976; Porter et al. 1981b).

Tryptic digestions of cytosolic or mitochondrial aspartate aminotransferase were performed on protein samples (67 and 150 mg, respectively) that had been borohydride-reduced and carboxymethylated with radioactive iodoacetate (Barra et al. 1984; Martini et al. 1984). Trypsin was added in two aliquots to a final E/S ratio of 1/30 and incubated for 5 h at 37°C in 0.1 M ammonium bicarbonate. The peptides were initially fractionated by gel filtration through a column (2.5 cm × 115 cm) of Sephadex G-25 superfine equilibrated with 10% acetic acid. The first fractions from this column were further fractionated by gel filtration through a column (2.5 cm × 115 cm) of Sephadex G-50 fine using the same solvent. Final purification of peptides was by high-performance liquid chromatography (HPLC) on a macroporous reverse-phase column (Brownlee Labs, RP-300, 10 μm) with gradients of acetonitrile in 0.2% (v/v) trifluoroacetic acid generated using a Beckman Model 420 instrument at a flow rate of 1.0 ml/min. The absorbance of the effluent was monitored at both 220 nm and either 280 or 325 nm using a Beckman Model 165 variable wavelength detector.

For the CNBr cleavages, 10 mg native mitochondrial aspartate aminotransferase was treated with an equal weight of reagent in 70% formic acid for 15 h at room temperature in the dark. After repeated lyophilization the peptide mixture was fractionated by HPLC on a preparative macroporous column (0.7 cm × 25 cm) from Brownlee Labs. The sample was dissolved in 0.75 ml 0.2% trifluoroacetic acid and loaded onto the column in five aliquots. Elution was performed using a gradient of 20% to 75% acetonitrile in 0.2% trifluoroacetic acid developed over 32 min.

For the cytosolic isoenzyme, 40 mg protein was carboxymethylated and then treated with CNBr under the conditions described above. The peptide mixture was fractionated by gel filtration through a column (2.5 cm × 135 cm) of Sephadex G-50 superfine and the fractions of interest were further purified by HPLC.

The manual dansyl-Edman procedure was used for determination of sequences of peptides, supplemented where necessary by digestion with carboxypeptidases followed by analysis of the released amino acids; details of these procedures have been reported previously (Barra et al. 1984).

Amino acid sequences were compared using the computer program described by Lipman and Pearson (1985), a copy of which was kindly provided by those authors. The program produces the best alignment of the two sequences compared, leaving gaps where necessary. In addition, it classifies amino acid substitutions between two sequences as conservative or nonconservative based on the PAM 250 replaceability matrix of Dayhoff (1978).

Quantitative comparisons between sequences were done in terms of the average number of amino acid substitutions per site (K_{aa}) according to Kimura (1983). The values of K_{aa} were calculated from the empirical relationship

$$K_{aa} = -\ln(1 - p_d - \tfrac{1}{5}p_d^2)$$

where p_d is the fraction of amino acid sequence differences (out of the total number of amino acids in each sequence) between the two proteins compared. Where the two proteins compared were of different lengths, gaps in the shorter sequence were counted as mismatches and p_d was calculated on the basis of the number of residues in the longer sequence.

Results and Discussion

Amino Acid Sequences of Cytosolic and Mitochondrial Aspartate Aminotransferases from Horse Heart

Substantial portions (88% and 94%, respectively) of the amino acid sequences of the cytosolic (Martini et al. 1984) and mitochondrial (Martini et al. 1983) aspartate aminotransferases from horse heart have previously been reported. We have now analyzed tryptic peptides and some cyanogen bromide fragments from the two isoenzymes. The results obtained both confirm the previous partial structures and allow completion of these structures. Details of peptide separation and sequence analysis have not been given here since the tactics used were very similar to those employed for determination of the structure of the mitochondrial isoenzyme from human heart (Martini et al. 1985). Table 1 summarizes which amino acid residues were previously unas-

Table 1. Peptides analyzed to identify amino acid residues previously unassigned in the sequences of cytosolic and mitochondrial aspartate aminotransferases from horse heart

Isoenzyme	Residues	Peptides analyzed
Cytosolic	56–70, 77–79	Trypsin 56–59, 60–80
	119–123	Trypsin 114–121, 122–129
	159–161	Trypsin 157–166
	184–188	*Staphylococcus aureus* protease 183–203 from trypsin 167–206
	213–216	CNBr 213–287
	317–319	Trypsin 305–317, 318–324
	351–360	Trypsin 346–359 from CNBr 334–359; CNBr 360–389
	381	Trypsin 378–386
	400–412	CNBr 390–412; Trypsin 396–410, 411–412
Mitochondrial	22–33	*S. aureus* protease 21–31 from CNBr 10–31; trypsin 31–39
	141–149	Trypsin 131–141, 142–151
	263–264	Trypsin 259–267

signed and gives information on the peptides analyzed to complete the sequences. The complete sequences of the cytosolic and mitochondrial isoenzymes are given in Figs. 1 and 2, respectively.

A few points about the sequences require specific comment. The penultimate residue of the cytosolic isoenzyme was confirmed as phenylalanine by digestion of the C-terminal cyanogen bromide fragment with carboxypeptidase Y, and this identification was consistent with isolation of a peptide Phe–Gln from the tryptic digest of the intact protein. Previously (Bossa et al. 1981) we had found valine at this position by carboxypeptidase digestion of the intact protein. It is important to note, however, that the samples of aspartate aminotransferase used in this and in the previous work were isolated from different hearts and hence it seems probable that two variants of the enzyme exist that differ at position 411. Microheterogeneity has previously been reported for the human mitochondrial isoenzyme (Martini et al. 1985) and for the pig cytosolic isoenzyme (Doonan et al. 1975); in the latter case, the variable region was also at the C-terminus and involved a deletion of residues 404–406.

For the mitochondrial isoenzyme (Fig. 2) residue 276 has been reassigned as glutamic acid rather than glutamine; this assignment was confirmed by direct identification by HPLC of the phenylthiohydantoin-amino acid released during dansyl-Edman sequence analysis (Simmaco et al. 1985). The identity of this residue was reexamined in light of the recent report by Joh et al. (1985) of the base sequence of the cDNA for mitochondrial aspartate aminotransferase from pig liver, which showed glutamic acid at position 276, contrary to our previous assignment (Barra et al. 1980). Having reexamined the residue at this position in the human isoenzyme, we again find glutamic acid. Hence residue 276 is invariant in all of the mitochondrial aspartate aminotransferases so far examined.

Interspecies Comparisons of the Sequences

Figures 1 and 2 give comparisons of the sequences of all aspartate aminotransferases reported to date [with the exception of that from *Escherichia coli* (Kondo et al. 1984)], showing only those residues that differ from those in the corresponding isoenzymes from horse heart. For the cytosolic isoenzymes, 80.3% of the residues are invariant in the three sequences known. Substitutions are unequally distributed throughout the sequences, being particularly numerous in the first 16 residues (44% of residues identical) and in the region of residues 308–321 (50% of residues identical). It is noteworthy that whereas the mammalian isoenzymes are identical for residues 271–281, the chicken isoenzyme differs from them in 64% of its residues. Also of interest is that the N-terminal amino acids of the horse and chicken isoenzymes are acetylated, whereas that of the pig isoenzyme is not. All substitutions between the pig and horse isoenzymes are conservative with the exception of that at position 308 (phenylalanine for arginine); this region also contains the greatest concentration of nonconservative changes (positions 308, 314, and 317) when the chicken isoenzyme is compared with the isoenzymes of mammals.

Among the mitochondrial isoenzymes (Fig. 2) 81% of the positions are occupied by identical residues. [Note that at position 11 of the rat isoenzyme Huynh et al. (1980) give glycine, whereas we (Bossa et al. 1981) find proline; the residue is given as proline in Fig. 2, consistent with all other mitochondrial isoenzymes.] At four positions (22, 162, 234 and 341), the horse isoenzyme differs from the other four isoenzymes whose structures are known. Again, substitutions are unequally distributed, being particularly few in the N-terminal region (4% differences in the first 48 residues). This is in marked contrast to the situation with the cytosolic isoenzymes; the

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                                    30                                  60
Horse     *TSPSIFVEVPQAQPVLVFKLTADFREDPDPRKVNLGVGAYRTDDCQPWVLPVVRKVEQKI
Pig        AP  V A              I                                      R
Chicken   *AA   AA  R P  A           G S              EG                L

                                    90                                  120
Horse     ANNSSLNHEYLPILGLAEFRSCASRLALGDDSPALQEKRVGGVQSLGGTGALRIGAEFLS
Pig                            T                                       A
Chicken    G G             P   AN   I        IAQ    S  G               -

                                    150                                 180
Horse     RWYNGTNNKNTPVYVSSPTWENHNGVFSGAGFKDIRSYHYWDATKRGLDLQGFLNDLENA
Pig               D                TT         R   TE           S
Chicken        N  TA               S  MD       T R    A        L S M K

                                    210                                 240
Horse     PEFSIFVLHACAHNPTGTDPTPEQWKQIASVMKRRFLFPFFDSAYQGFASGNLDRDAWAV
Pig                                                              EK    I
Chicken         I                DE     A     C                 EK

                                    270                                 300
Horse     RYFVSEGFELFCAQSFSKNFGLYNERVGNLTVVAKEPDSILRVLSQMQKIVRITWSNPPA
Pig                                                              V
Chicken                             S  G DE NVQ      E    T      S

                                    330                                 360
Horse     QGARIVAFTLSDPGLFKEWTGNVKTMADRILSMRSELRARLEALKTPGTWNHITEQIGMF
Pig             R     E  H                                         D
Chicken         T  TS Q  A  KD        V L      S   S G             D

                                    390                   412
Horse     SFTGLNPKQVEYLVNQKHIYLLPSGRINMCGLTTKNLDYVATSIHEAVTKFQ
Pig                  I                                      I
Chicken             MIKE     MA                    K        I
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Fig. 1. Amino acid sequences of cytosolic aspartate aminotransferases. For the isoenzymes from pig (Barra et al. 1980) and chicken (Shlyapnikov et al. 1979), only those amino acids that differ from those in the horse sequence are shown. The isoenzyme from chicken is 2 amino acid residues shorter than the others (deletions at positions 1 and 120). Asterisks indicate that the N-terminal residues of the isoenzymes from horse and chicken are acetylated

possible significance of high conservation of the N-terminal region of the mitochondrial isoenzymes has recently been discussed (O'Donovan et al. 1985). Regions of high variability also occur. For example, among residues 299–313 only 53% are identical and three of the substitutions (at positions 300, 310, and 313) are nonconservative.

Comparison of the Cytosolic and Mitochondrial Aspartate Aminotransferases from Horse Heart

The cytosolic and mitochondrial aspartate aminotransferases from horse heart are compared in Fig. 3. The mitochondrial isoenzyme is 11 residues shorter than the cytosolic form, and the best alignment is obtained by leaving gaps in the former at positions 1, 2, 64, 124–127, 153, 407, 411, and 412. Maximum similarity is obtained by leaving an additional gap at position 253 in the mitochondrial isoenzyme and a corresponding gap between residues 250 and 251 in the cytosolic form. Aligned in this way the sequences show 48.1% identity of structure based on the longer sequence. Conservative substitutions occur at 35.2% of the positions, and the remaining 16.7% are nonconservation changes.

Rates of Evolution of the Aspartate Aminotransferases

Each of the amino acid sequences in Figs. 1 and 2 was compared with all the others and also with the sequence of the enzyme from *E. coli* (Kondo et al. 1984). In each case the percentage sequence difference was calculated based on the longer of the two sequences (if different in length); the resulting values are given in Table 2. For each comparison, the average number of amino acid substitutions per site (K_{aa}; Kimura 1983) was calculated; these values are shown in parentheses in Table 2. The K_{aa} values provide estimates of the total number of substitutions that have occurred between two sequences, including multiple substitutions at a single site.

Table 3 gives average K_{aa} values for various groups of comparisons. Examination of these values allows preliminary conclusions to be drawn about rates of evolution of aspartate aminotransferases.

First, the results reported here allow the first di-

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                                    30                                  60
Horse     SSWWAHVEMGPPDPILGVTEAYKRDTNSKKMNLGVGAYRDDNGKPYVLPSVRKAEAQIAA
Rat            T                F                                    G
Pig                             F
Human          T                F
Chicken        S                F                          NC        M

                                    90                                 120
Horse     KNLDKEYLPIGGLAEFCKASAELALGENSEALKSGRYVTVQSISGTGALRIGANFLQRFF
Rat                        D                 V     F    T         V  S
Pig                                        N V          T
Human                                        V     F    T            S
Chicken   KM        A   D TR                 F          G     S  V

                                   150                                 180
Horse     KFSRDVFLPKPSWGNHTPIFRDAGLQLHAYRYYDPKTCGFDVTGALEDISKIPQQSIILL
Rat                  G    E A    M  EG             FS           E  VL
Pig                              M   S             F            A  V
Human              T             M  QG             F   V        E  VL
Chicken         Y                  E Q           SL F   M        EK

                                   210                                 240
Horse     HACAHNPTGVDPRPEQWKEIATLVKKNNLFAFFDMAYQGFASGDGNKDAWAVRYFIEQGI
Rat                         M AY                                   H
Pig                         M                                      H
Human                          V   R                               H
Chicken                 E     L SV   R  L Y            I R    L H

                                   270                                 300
Horse     NVCLCQSYAKNMGLYGERVGAFTMVCKDADEAKRVESQLKILIRPLYSNPPLNGARIAST
Rat                               V     E                           A
Pig                               V     E               M     V
Human                                                   M           AA
Chicken   D V S             A     VI R  E               M     M       L

                                   330                                 360
Horse     ILTSPDLRKQWLQEVKGMADRIISMRTQLVSNLKKEGSSHSWQHIADQIGMFCFTGLKPE
Rat             QG             G                  N    T          I
Pig             Q                                 N    V          I
Human      NT                   G              T N    T
Chicken    NT E   E  V                           N    T

                                   390        401
Horse     QVERLTKEFSIYMTKDGRISVAGVTSGNVGYLAHAIHQVTK
Rat
Pig
Human          I                    S
Chicken                           A S
```

Fig. 2. Amino acid sequences of mitochondrial aspartate aminotransferases. For the isoenzymes from rat (Huynh et al. 1980), pig (Barra et al. 1980), human (Martini et al. 1985), and chicken (Graff-Hausner et al. 1983), only those amino acids that differ from those in the horse sequence are shown. See the text for specific references to residue 11 of the rat sequence and to residue 276 of the pig and human sequences

rect comparison of the sequences of two mammalian cytosolic isoenzymes. The value of K_{aa} obtained (0.064) is very close to the average (0.069) for comparisons of all the known mammalian mitochondrial isoenzymes. This suggests that the overall rates of evolution of mitochondrial and cytosolic aspartate aminotransferases have been very similar in the various mammalian orders, although testing of this conclusion will require determination of more sequences of cytosolic isoenzymes. This result is in direct conflict with those from immunochemical comparisons (Sonderegger and Christen 1978), which showed that the immunological distances among mammalian cytosolic aspartate aminotransferases are twice as great as among the mammalian mitochondrial forms. This discrepancy has been rationalized on the basis of high conservation of restricted, antigenic regions of the mitochondrial isoenzymes (Doonan et al. 1984b).

It should be noted that comparisons of the incomplete sequences of the isoenzymes from horse heart with those from pig (Martini et al. 1983, 1984) were somewhat misleading in suggesting greater differences between the cytosolic (5.3%) than between the mitochondrial (3.7%) isoenzymes; the results presented here show that the partial sequences were not truly representative of the total structures, as Martini et al. (1984) suspected might be the case.

Fig. 3. Comparison of the amino acid sequences of cytosolic and mitochondrial aspartate aminotransferases from horse heart. The alignment was obtained using the computer program described by Lipman and Pearson (1985). Double dots and single dots between the sequences indicate identical residues and conservative substitutions, respectively. The numbering system used is that of the cytosolic isoenzyme

Table 2. Quantitative comparisons of the amino acid sequences of aspartate aminotransferases

	% Sequence difference (100 × average substitutions/site)							
	Horse {m}	Human {m}	Rat {m}	Chicken {m}	Pig {c}	Horse {c}	Chicken {c}	*E. coli*
	4.7	6.7	5.7	13.7	52.7	53.4	51.7	59.6
Pig {m}	(4.9)	(7.0)	(5.9)	(15.2)	(87.4)	(89.4)	(84.5)	(110)
		7.2	8.2	13.7	51.5	51.9	50.5	60.3
Horse {m}		(7.6)	(8.7)	(15.2)	(83.9)	(85.1)	(81.2)	(113)
			7.0	13.5	51.2	51.7	50.5	60.1
Human {m}			(7.4)	(14.9)	(83.1)	(84.5)	(81.2)	(112)
				15.2	53.2	53.6	51.5	60.1
Rat {m}				(17.0)	(88.8)	(90.0)	(83.9)	(112)
					53.9	54.4	53.9	59.6
Chicken {m}					(90.9)	(92.4)	(90.9)	(110)
						6.1	17.5	60.9
Pig {c}						(6.4)	(20.0)	(115)
							18.4	61.0
Horse {c}							(21.2)	(115)
								60.7
Chicken {c}								(114)

Percentage sequence differences are based on the longer of the two sequences compared, taking deletions in the shorter sequence as mismatches. Average substitutions/site are given in parentheses and were calculated as described in the text. The abbreviations "{m}" and "{c}" denote mitochondrial and cytosolic, respectively

It is interesting to enquire whether the mammalian mitochondrial isoenzymes have evolved at a constant rate in the various lineages. Kimura (1983) has presented a method for testing constancy in various lineages of rates of evolution from a common ancestor on the assumption that the amino acid substitutions follow the Poisson distribution. In the present case the ratio of the observed variance of differences between sequences to that expected by chance is 1.45. From the distribution of χ^2 it is found

Table 3. Average K_{aa} values for groups of comparisons taken from Table 2

Comparison	100 × average $K_{aa} \pm SD$
All mammalian mitochondrial isoenzymes	6.9 ± 1.3
Pig cytosolic against horse cytosolic	6.4
Mammalian mitochondrial against chicken mitochondrial isoenzymes	15.6 ± 1.0
Mammalian cytosolic against chicken cytosolic isoenzymes	20.6 ± 0.8
All cytosolic against all mitochondrial isoenzymes	86.5 ± 3.7
E. coli against all animal isoenzymes	113 ± 1

that that ratio does not differ significantly from unity. Given that the points of divergence of the four lineages compared are not strictly the same, the ratio is an overestimate and the results strongly support a constant rate of evolution.

The absolute rate of evolution of the mammalian mitochondrial isoenzymes in terms of amino acid substitutions per site per year (k_{aa}) can be calculated from $k_{aa} = K_{aa}/2T$, where T is the time elapsed since divergence of the mammals from their common ancestor. Taking $T = 80 \times 10^6$ years, $k_{aa} = 0.43 \times 10^{-9}$/year. This compares with values of 0.3×10^{-9}/year for cytochrome c and 1.2×10^{-9}/year for the α-chain of hemoglobin (Kimura 1983). Hence aspartate aminotransferase is a relatively slowly evolving protein.

The situation for the chicken isoenzymes is problematic. The average K_{aa} value for the comparison between chicken and mammalian mitochondrial isoenzymes (0.156) is somewhat smaller than that (0.206) for the cytosolic forms. This may indicate that in chicken the cytosolic isoenzyme has evolved more rapidly than the mitochondrial form, but more data are required if we are to investigate this possibility. Furthermore, the absolute rates of evolution of the chicken isoenzymes appear to be lower than those of the mammalian forms. Taking $T = 300 \times 10^6$ years for the divergence between mammals and birds, the k_{aa} values for the mitochondrial and cytosolic isoenzymes are 0.26×10^{-9}/year and 0.34×10^{-9}/year, respectively. This may show that the rate of evolution of aspartate aminotransferases has not remained constant over the extended time since the divergence between mammals and birds; a similar observation has been made with reference to the α-chain of hemoglobin (Kimura 1983). Alternatively, the accepted divergence time between birds and mammals may be in error; a value of 200×10^6 years would give k_{aa} values for the comparison of chicken and mammalian aspartate aminotransferases in line with the k_{aa} for comparisons within the mammals. However, given that apparently well-established cases of inconstancy of evolutionary rates have been reported (e.g., Goodman 1985; Lee et al. 1985), the former possibility seems the more likely.

Given in Table 2 are the percentage differences and K_{aa} values for comparisons between all mitochondrial isoenzymes and all cytosolic isoenzymes. The interspecies comparisons are legitimate, since the gene duplication that gave rise to the two isoenzymes predated divergence of the species considered. The K_{aa} values are very similar for all comparisons, with an average value of 0.865. If it is assumed that the rates of evolution of the two isoenzymes have been constant and equal since the gene duplication that gave rise to them occurred, then the elapsed time can be calculated from the rate of evolution of the mammalian mitochondrial forms (see above). The value obtained is 10^9 years. This is consistent with the date estimated for emergence of the eukaryotes—about 1.3×10^9 years ago, when oxygen became abundant in the Earth's atmosphere (Dobzhansky et al. 1977).

Finally, Table 2 gives the results of comparisons between the enzyme from *E. coli* (Kondo et al. 1984) and each of the animal isoenzymes. The K_{aa} values are very similar for both cytosolic and mitochondrial isoenzymes, with an average of 1.13 (Table 3). Hence at the level of overall sequence the cytosolic and mitochondrial isoenzymes are equally related to the prokaryotic enzyme. This is not consistent with a model in which the cytosolic enzyme originated in a protoeukaryote and the mitochondrial enzyme was introduced by a symbiotic prokaryote. Rather, it suggests that both isoenzymes originated by a gene duplication in the early eukaryotic cell, and that one of the protein products was sequestered into the mitochondria. Again assuming a constant rate of evolution of the prokaryotic and animal isoenzymes, the time since the last common ancestor of the eukaryotic and prokaryotic enzymes can be estimated as 1.3×10^9 years.

The dates given above for the gene-duplication event and, more particularly, for divergence of the genes for prokaryotic and eukaryotic aspartate aminotransferases must both be considered lowest estimates. Both rely on the assumption that the K_{aa} values as calculated fully allow for multiple replacements at single sites in the isoenzymes; for more distantly related sequences this assumption is unlikely to be valid, given the simple type of Poisson correction used. In addition both are calculated from the rate of evolution of the mammalian isoenzymes. As outlined above, the comparisons between mammalian and chicken isoenzymes raise the possibility that evolution may have been faster in mammalian lineages than in some others. For resolution of these points it would be of interest to have sequences of

aspartate aminotransferases from animals more distantly related than are birds and mammals.

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