

## Negative Charge Correlates with Neural Expression in Vertebrate Aldolase Isozymes

T.J.S. Merritt,\* J.M. Quattro

Department of Biological Sciences, Program in Marine Science, Baruch Institute and School of the Environment, University of South Carolina, Columbia, SC 29208, USA

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**Abstract.** Electrophoretic studies suggest that negatively charged neural proteins are a general feature of jawed vertebrates. In an apparent example of this, teleost fish express three aldolase isozymes, one of which is expressed predominantly in neural tissues and is more negatively charged than its more generally expressed paralogues. We characterized three aldolase isozymes from a single species of teleost fish, zebrafish (*Danio rerio*). These sequences indicated that the correlation of net negative charge and neural expression suggested in other species by gel electrophoresis was supported by sequence analysis. When aldolase sequences from the databases were included in phylogenetic analyses, the negative charge/neural expression phenomenon was observed across the gnathostome vertebrate sequences examined. We found no evidence for a period of positive Darwinian selection resulting in an accumulation of negatively charged amino acids during the evolution of the neural aldolase isozymes. This is likely attributable, however, to limitations associated with the age of the duplication responsible for the neural isozyme and the reconstruction of ancestral sequences.

**Key words:** Fructose biphosphate aldolase — Gene duplication — Neural expression — Teleost fish

### Introduction

Individual tissues often express unique sets of proteins. For example, the proteins of the vertebrate eye and brain are, as a group, more negatively charged than those proteins expressed in other tissues (Moore and McGregor 1965; Moore 1973). This charge bias has been well characterized in teleost fish, in which the isozymes [distinct forms of enzymes that catalyze identical chemical reactions (Markert and Moller 1959)] of the eye and brain of a number of gene families are more negatively charged than their paralogues expressed in other tissues (e.g., Fisher et al. 1980; Morizot and Schmidt 1990; Merritt and Quattro 2001). The apparent correlation between net negative charge and neural expression has led to the suggestion that the negative charge of neural proteins might be an adaptation to the neural environment (Fisher et al. 1980; Merritt and Quattro 2001).

We recently examined the evolution of a negatively charged, neurally expressed, isozyme of triosephosphate isomerase (TPI; EC 5.3.1.1) in teleost fish (Merritt and Quattro 2001). Two TPI proteins are expressed in teleost fish, including a neutral, generally expressed, isozyme (TPI-B) and a negatively charged, neurally expressed, isozyme (TPI-A) (Pontier and Hart 1981; Morizot and Schmidt 1990). All other

Sequences reported in this paper have been submitted to GenBank (accession numbers AF533645–AF533647)

\*Current address: Department of Ecology and Evolution, State University of New York—Stony Brook, Stony Brook, NY 117794, USA

Correspondence to: Joseph M. Quattro; email: quattro@mail.biol.sc.edu

vertebrates express only a single TPI protein (Maquat et al. 1985; Old and Mohrenweiser 1988; Cheng et al. 1990; Straus and Gilbert 1985; Kuraku et al. 1999). The two teleost TPI isozymes are the products of separate loci (Pontier and Hart 1981) that result from a duplication event early in the radiation of teleost fish (Merritt and Quattro 2001). Importantly, the negative charge of the teleost neural TPI isozyme results from the biased accumulation of negatively charged amino acids during a period of positive Darwinian selection (Merritt and Quattro 2001).

Negative neural isozymes are not restricted to teleost fish but are found across gnathostome (jawed) vertebrates. For example, the vertebrate creatine kinase (Richardson et al. 1986; Morizot and Schmidt 1990) and enolase (Morizot and Siciliano 1984; Marangos and Schmechel 1987; Morizot and Schmidt 1990; Tracy and Hedges 2000) gene families include a negatively charged neural isozyme. The presence of negatively charged neural isozymes across a variety vertebrate taxa suggests that the selective pressures favoring the accumulation of negative amino acids by teleost neural isozymes [e.g. TP1 (Merritt and Quattro 2001)] might be present across all vertebrate taxa.

To address this possibility, we examined the evolutionary history of the aldolase (class I fructose-1,6-bisphosphate aldolase; EC 4.1.2.13) gene family, which includes a negatively charged neural isozyme common to gnathostome vertebrates (e.g., Penhoet et al. 1967; Phillipp et al. 1979; Morizot and Schmidt 1990). Gnathostome vertebrates express three aldolase isozymes: aldolase A is expressed predominantly in muscle, aldolase B is expressed predominantly in the liver, and aldolase C is expressed predominantly in the brain and nervous system (Penhoet et al. 1967; Phillipp et al. 1979; Morizot and Schmidt 1990). Gel electrophoresis indicated that both aldolase A and aldolase B are positively charged, whereas aldolase C is negatively charged (Penhoet et al. 1967; Phillipp et al. 1979; Morizot and Schmidt 1990). Jawless vertebrates (agnathans; lamprey and hagfish) express two aldolase isozymes; neither is characteristically neural in expression and both are neutrally charged (R. Zhang et al. 1997). The three aldolase isozymes in gnathostomes and the two isozymes in agnathans share a single-gene ancestor early in the radiation of vertebrates (Kuraku et al. 1999); this suggests that the different number of isozymes in each group result from lineage-specific gene duplications.

All three aldolase isozymes have been sequenced from a number of tetrapod vertebrates [e.g., human (Sakakibara et al. 1985; Besmond et al. 1983; Rottmann et al. 1987) and rat (Mukai et al. 1986, 1991; Tsutsumi et al. 1984)]. Aldolase B (Llewellyn et al. 1995) and aldolase C (Berardini et al. 1997) have been described from teleost fish but, unfortunately, from

two phylogenetically disparate groups. All three aldolase loci have not been described from a single taxonomic group of fish. Given that isozymes are known to vary in charge between even very closely related species (e.g., Pointer and Hart 1979, 1981), comparisons of currently available teleost aldolase sequences risk confounding between-locus patterns of enzyme diversification with between-species patterns. To circumvent the potential for confounding comparisons, we report the complete coding sequence of the three aldolase isozymes from a representative teleost fish, zebrafish (*Danio rerio*). We use these sequences and other aldolase sequences from GenBank in phylogenetic analyses to examine the evolutionary history of vertebrate neural aldolase proteins.

## Methods

*Danio rerio* (zebrafish) tissues were dissected from single fish purchased locally and processed immediately or stored at  $-70^{\circ}\text{C}$  for later processing. Total RNA was purified from tissues using RNeasy (Qiagen). Complementary DNA (cDNA) was synthesized from total RNA using the Superscript Preamplification System (Gibco BRL).

### Aldolase Cloning and Sequencing

Oligonucleotide primers were designed for individual aldolase loci using alignments of zebrafish expressed sequence tags (ESTs) and other vertebrate aldolase sequences available from GenBank.

**Aldolase A.** Partial sequences of the 5' and 3' ends of the zebrafish *Ald-A* mRNA are available through GenBank (GenBank accession numbers AI436937 and AA606169, respectively). These sequences were used to design specific oligonucleotide primers that flanked the aldolase A coding region.

DrALDA5': 5'-ACG TGG TCG AGG CTG ATC CGT G-3'  
DrALDA3': 5'-CAG GTT GAG TGA TTC TTC AGG-3'

These two primers were used to amplify a 1230-base pair (bp) segment of the *Ald-A* mRNA from zebrafish muscle cDNA by the polymerase chain reaction (PCR) (Saiki et al. 1988). PCR was carried out for 40 cycles under the following conditions: denaturation at  $95^{\circ}\text{C}$  for 1 min, annealing at  $48^{\circ}\text{C}$  for 1 min, and extension at  $72^{\circ}\text{C}$  for 1 min. PCR products were cloned into pGEM T-vector (Promega) and sequenced using a 377 automated Sequencer (Perkin Elmer). Dye terminator sequencing was performed with BigDye dye termination mix (Perkin Elmer Biosystems) using the manufacturer's suggested protocol. At least three independent clones per PCR fragment were sequenced on both strands.

**Aldolase B.** EST sequences (GenBank accession numbers AI878510, AI629370, AI522695, AI477716, AI477428) provided partial 3' sequences of the zebrafish *Aldolase-B* mRNA and were used to design an oligonucleotide primer specific to this region.

DrALDB3'R: 5'-ATA ACT TTG GCA TCT TCA CTC-3'

An alignment of all vertebrate aldolase genes in GenBank was used to design a primer near the 5' end of the aldolase coding region.

ALD 44F: 5'-CCN GAR CAR AAR AAR GA-3'

where N = A,G,C,T and R = A,G. Numbers refer to the amino acid position occupied by the 3' base of the primer in the sea bream (*Sparus aurata*) aldolase B (Llewellyn et al. 1995). These two primers were used to amplify a 1142-bp product from zebrafish

liver cDNA using PCR conditions stated above. The extreme 5' coding and untranslated region of the zebrafish aldolase B cDNA was amplified using the RACE method (Frohman 1990). RACE amplifications used gene-specific primers designed from the initial gene fragment. Conditions for PCR, cloning, and sequencing were as described above.

**Aldolase C.** A number of zebrafish ESTs deposited in GenBank have been identified as fragments of the *Ald-C* mRNA, presumably by sequence similarity scores. However, our preliminary phylogenetic analyses indicated that these fragments were most likely aldolase B and not aldolase C as reported (data not shown). To amplify zebrafish aldolase C, degenerate oligonucleotide primers were designed using an alignment of all vertebrate aldolase C genes in GenBank (excluding the zebrafish ESTs).

ALDC 363R: 5'-TAN GCR TGR TIN MCN AYR TA-3'

ALDC 301R: 5'-TAN GAR AAN GTN ARN GCC CA-3'

where N = A,G,C,T, R = A,G, M = A,C, and Y = C,T, and numbers refer to the amino acid position occupied by 3' base of each primer relative to the predicted sea bream aldolase B (Llewellyn et al. 1995).

PCR amplification using primers ALD 44F and ALDC 363R was performed (conditions as described above) on zebrafish brain cDNA but consistently yielded only faint PCR products on agarose gels. This product was diluted 1:100 and used as template in a partially nested PCR reaction using primers ALD 44F and ALDC 301R, yielding an 879-bp product. The extreme 5' and 3' coding and untranslated regions of this sequence were amplified using the RACE method as described above.

Isoelectric point (pI) values were calculated for each predicted amino acid sequence using the EMBL Isoelectric Point service (<http://www.embl-heidelberg.de/cgi/pi-wrapper.pl>).

## Phylogenetic Analyses

In addition to the three zebrafish aldolase sequences reported here, the following sequences were obtained from GenBank and used in phylogenetic analyses: human aldolase A (GenBank accession number M1 1560), human aldolase B (K01 177), human aldolase C (X05196), rat aldolase A (M12919), rat aldolase B (M10149), rat aldolase C (M63656), sea bream aldolase B (X82278), pufferfish aldolase C (AF041454), goldfish aldolase C (U36777), lamprey muscle aldolase (D38620), lamprey nonmuscle aldolase (D38619), amphioxus aldolase (AB005035), nematode aldolase 1 (D83738), and nematode aldolase 2 (D83739). Complete sequences are also available for the coding regions of the three aldolase isozymes from the frog *Xenopus leavis*. *Xenopus leavis* is, however, known to be a recent tetraploid (Kobel and DuPasquier 1986). To avoid confounding gene with genome duplication, the *Xenopus* aldolase sequences were not included in this analysis.

Amino acid sequences were aligned using the ClustalW multiple sequence alignment computer program using the default settings (Thompson et al. 1994). Minor adjustments were made to the alignment manually, resulting in an overall alignment of 364 amino acid positions. Nucleotide sequences were then aligned using the amino acid alignment as a guide. A phylogeny was constructed by the neighbor-joining (NJ) method (Saitou and Nei 1987) using the algorithm implemented in MEGA [version 1.0 (Kumar et al. 1993)]. Pairwise distances were calculated from first- and second-position nucleotides using the Kimura (1980) two-parameter model, and the complete deletion option for gaps and missing data. Bootstrapping (Felsenstein 1985) was used to evaluate the degree of support for particular groupings in the NJ analysis.

We estimated the numbers of synonymous ( $b_S$ ) and nonsynonymous ( $b_N$ ) substitutions per site for each branch of the tree using the BN-BS computer program and the methods of Zhang

et al. (1998). Values reported were calculated using proportional differences to calculate the length of each branch. Using the Jukes-Cantor (1969) method of distance calculation gave similar results (data not shown). The transition-transversion ratio needed for this method was calculated using MEGA [version 1.0 (Kumar et al. 1993)]. Ancestral sequences were also calculated for each node of the tree using the distance-based method of Zhang et al. (1998). Reconstructions used the ANC-GENE computer program (Zhang et al. 1998) with the Jones, Taylor, and Thorton (JTT) (Jones et al. 1992) model of amino acid substitution. Reconstruction of ancestral sequences using a Poisson model of amino acid substitution, or parsimony, gave essentially the same results (data not shown). Amino acid 348 was not included in the ancestral reconstruction because of an alignment gap (see Fig. 1).

## Results

### Cloning

PCR amplification from zebrafish muscle cDNA using the DrALDA5'F and DrALDA3'R primers yielded a single product, 1230 nucleotides in length, including an ATG start codon, a TAA stop codon, 75 nucleotides of 5' untranslated sequence, and 60 nucleotides of 3' untranslated sequence. The predicted open reading frame (ORF) codes for a 364-amino acid protein with a predicted isoelectric point of 8.2 (Fig. 1). This sequence was identical in overlapping regions to zebrafish aldolase A ESTs identified in GenBank (hereafter we refer to this sequence as DrALD-A).

PCR amplifications using zebrafish liver cDNA as template and the ALD 44F and DrALDB3'R primers yielded a single product of 1142 nucleotides. This product was identical to the zebrafish aldolase B EST partial sequences across overlapping regions. When combined with sequence produced from 5' RACE amplifications using gene-specific primers, a mRNA was assembled comprising 1189 nucleotides, including an ATG start codon, a TAA stop codon, 28 nucleotides of 5' untranslated sequence, and 66 nucleotides of 3' untranslated sequence. An ORF codes for a 364-amino acid protein with a predicted isoelectric point of 8.2 (Fig. 1). This sequence is similar to other aldolase B sequences in GenBank (hereafter we refer to this sequence as DrALD-B).

PCR amplifications from zebrafish brain cDNA using the ALD44F, ALDC363R, and ALDC301R primers yielded a product of 813 nucleotides. When combined with sequence from 5' and 3' RACE amplifications, a 1477-nucleotide mRNA could be assembled containing an ATG start codon, a TGA stop codon, 201 nucleotides of 5' untranslated sequence, and 184 nucleotides of 3' untranslated sequence. An ORF codes for a 363-amino acid protein with a predicted isoelectric point of 6.2 (Fig. 1). This sequence is similar to other aldolase C genes in GenBank (Fig. 1; hereafter we refer to this sequence as DrALD-C).

human B	MAHRFPALTQ	EQKKELSEIA	QSIVANGKGI	LAADESVGTM	GNRLQRIKVE	NTEENRRQFR	60
rat B	.....S	.....	.R.....	.....	.....	.....	
sea bream B	.T.Q...S.SP	.....D..	.R...P....	.....T...	.K.F.N.N..	.I.....C..	
DrALD-B	.T.Q....ST	.....AT..	ER...P....	.....T...	AK.F.K.N..	.....S..	
Human A	.PYQY...P	.....D..	HR...P....	.....T.SI	AK...S.GT.	.....FY.	
rat A	.P.PY...P	.....AD..	HR...P....	.....T.SI	AK...S.GT.	.....FY.	
DrALD-A	.P.AY.F..P	.....D..	.R...P....	.....T.SV	AK.F.S.NA.	.....LY.	
ALD-A/C	-P.QY...P	.....D..	.R...P....	.....T.S	AK...Q.G..	.....LY.	
human C	.P.SY...SA	.....D..	LR...P....	.....S	AK..SQ.G..	.....LY.	
rat C	.P.SY...SA	.....D..	LR...P....	.....S	AK..SQ.G..	.....LY.	
goldfish C	.T.QY...T	...R..QD..	.R...P....	.....T.S	AK..NP.G..	.....LY.	
pufferfish C	.T.QY...F.P	.....Q...	.R...P....	.....T.S	AK.FNP.G..	.....RY.	
DrALD-C	.T.QY...A	.....QD..	.R...P....	.....T.S	AK..NP.G..	.....LY.	
ALD-C	-P.QY...P	.....D..	.R...P....	.....T.S	AK..NQ.G..	.....LY.	
human B	EILFSVDSSI	NQSIGGVILF	HETLYQKDSQ	GKLFRNILKE	KGIVVGIKLD	QGGAPLAGTN	120
rat B	.L.....N..	S.....	.....	.....	.....	.....	
sea bream B	D...T.A..	ANCV...I.F.	.....S.N	...PQVV..	.....V.	K.T...M..D	
DrALD-B	DL...D..	SE.....	.....SDK	.V..PKVI.D	.....V.	K.T.G...D	
Human A	QL.LTA.DRV	.PC.....	.....ADD	.RP.PQVI.S	.G.....V.	K.VV.....	
rat A	QL.LTA.DRV	.PC.....	.....ADD	.RP.PQVI.S	.G.....V.	K.VV.....	
DrALD-A	QL..TA.DRV	KPC.....	.....TDD	.V.SDY...R.M.	.....V.	K.VV.....	
ALD-A/C	QL..TA.DRV	KKC.....F.	.....TDD	.VP.PKLI.D	.....V.	K.VV.....	
human C	QV...A.DRV	KKC.....F.	.....DN	.VP.VRTIQD	.....V.	K.VV.....D	
rat C	QV...A.DRV	KKC.....F.	.....DN	.VP.VRTIQ.	...L...V.	K.VV.....D	
goldfish C	QL..TA.ERM	DKC.....F.	.....ADD	.TP.AKMI.D	R.....V.	K.VV.....	
pufferfish C	QL..TA.QR.	DSC.....F.	.....TDD	.VP.SKLI.D	R.....V.	K.VV.....	
DrALD-C	QL...A.ER.	DKC.....F.	.....NTDD	.TN.AQLI.D	R.....V.	K.VV.....	
ALD-C	QL..TA.DRV	KKC.....F.	.....TDD	.VP.AKLI.D	.....V.	K.VV.....	
human B	KETTIQGLDG	LSERCAQYKK	DGVDFGKWRA	VLRIADQCPS	SLAIQENANA	LARYASICQQ	180
rat B	.....	.....	.....	...S.....	.....	.....	
sea bream B	...T.....	.....	..C..A...C	..K.S.G..F	A...A...V	.....M	
DrALD-B	G...T.....	.....	..C..A...C	..K.S.S...	A.G.A...V	.....	
Human A	G...T.....	.....	..A..A...C	..K.GEHT..	A...M...V	.....	
rat A	G...T.....	.....	..A..A...C	..K.GEHT..	...M...V	.....	
DrALD-A	G...T.....	.Y.....	..A..A...C	..K.TPTT..	R...I...V	.....M	
ALD-A/C	G...T.....	.....	..A..A...C	..K.SETT..	A...M...V	.....	
human C	G...T.....	.....	..A..A...C	..K.SERT..	A...L...V	.....	
rat C	G...T.....	.L.....	..A..A...C	..K.S.RT..	A...L...V	.....	
goldfish C	G...T.....	.....	..A..A...S	..K.SETS..	E...M...V	.....	
pufferfish C	G...T.....	.....	..A..A...C	..K.SSTT..	E...F...V	.....	
DrALD-C	G...T.....	.....	..A..A...S	..K.S.TT..	E...M...V	.....	
ALD-C	G...T.....	.....	..A..A...C	..K.SETT..	A...M...V	.....	
human B	NGLVPIVEPE	VIPDGDHDL	HCQYVTEKVL	AAVYKALNDH	HVYLEGTLK	PNMVTAGHAC	240
rat B	.....	.L.....	...S...	.....	.....	...L.....	
sea bream B	.....	IL.....Q	R...A....	.....S.	.....	...P..S.	
DrALD-B	.....	IL.....K	Q...A....	.....S.	.....	...P..S.	
Human A	..I.....	IL.....K	R.....	.....S.	.I.....	...P...	
rat A	..I.....	IL.....K	R.....	.....S.	.....	...P...	
DrALD-A	H.I.....	IL.....K	R.....	.....S.	.....	...P...	
ALD-A/C	..I.....	IL.....K	R.....	.....S.	.....	...P...	
human C	..I.....	IL.....K	R.....	.....S.	.....	...P...	
rat C	..I.....	IL.....K	R..F....	.....S.	.....	...P...	
goldfish C	..I.....	IL.....K	R.....	..C...S.	.....	...S.	
pufferfish C	..I..V....	IL.....Q	R.....	.....S.	.....	...S.	
DrALD-C	..I.....	IL...E...K	R.....	..C...S.	.....	...S.	
ALD-C	..I.....	IL.....K	R.....	.....S.	.....	...S.	

**Fig. 1.** Amino acid sequence alignment for vertebrate aldolase amino acid sequences and inferred ancestral sequences. Predicted isoelectric point (pI) values are shown at the 3' end of each protein. A/C indicates the inferred common ancestor of the aldolase A and aldolase C proteins. C indicates the inferred common ancestor of the aldolase C proteins. Negative amino

acid positions specific to aldolase C proteins are *highlighted*: the single aldolase C-specific negative amino acid shared by both tetrapods and teleost fish is in *bold-face* type, positions specific to teleost fish aldolase C's are marked with *asterisks*, symbol and the position specific to tetrapod aldolase C's is marked with a *pound* symbol.

human B	TKKYTPQVA	MATVTALHRT	VPAAVPGICF	LSGGMSEEDA	TLNLNAINLC	PLPKPWKLSF	300
rat B	.....S.	.....S.	.....S.	.....S.	.....YR.	...R.....	
sea bream B	P..F..QE..	.....R..	...S.....	...Q...E.	SIH.....QV	..HR....T.	
<i>DrALD-B</i>	.....LE..	.....R..	.....	...Q...E.	S.....M.QL	S.HR.....	
Human A	.Q.FSH.EI.	.....R..	..P..T..T.	...Q...E.	SI.....K.	..L...A.T.	
rat A	.Q.FSN.EI.	.....R..	..P...VT.	...Q...E.	SI.....K.	..L...A.T.	
<i>DrALD-A</i>	SQ.N..QEI.	.....R..	..P...VT.	...Q...E.	.....M.Q.	..HR..A.T.	
<b>ALD-A/C</b>	PI..S..EI.	.....R..	..P...VT.	...Q...E.	S.....Q.	..HR..A.T.	
human C	PI..S..EI.	.....R..	..P...VT.	...Q...E.	SF.....R.	...R..A.T.	
rat C	PI..S..EI.	.....R..	..P...VT.	...Q...E.	S.....R.	S..R..A.T.	
goldfish C	PT.FSNQEI.	.....R..	..P..T.VT.	...Q...E.	SI.....N.	..T...A.T.	
pufferfish C	PI..SS.E..	.....R..	..P..T.VR.	...Q...E.	S.....N.	..A...A.T.	
<i>DrALD-C</i>	PT..SS.EI.	.....R..	..P..S.VT.	...Q...E.	SV...S..N.	..A...P.T.	
<b>ALD-C</b>	PI..S..EI.	.....R..	..P...VT.	...Q...E.	S.....Q.	...R..A.T.	
human B	SYGRALQAVA	LAAWGGKAAN	KEATQEAFMK	RAMANCQAAK	GQYVHTGSSG	AASTQSLFTA	360
rat B	.....S.	.....	.K.....	..V.....Q	.....	.....	
sea bream B	.....S.	.....Q..D..	.A...QV.VT	..KI.GL.S.	.E.KPS..AD	Q..Q...Y..	
<i>DrALD-B</i>	.....S.	.S..K.Q...	.K.S.D..VT	..KI.SL.S.	.E.KPS.QA.	Q.....	
Human A	.....S.	.K.....KE.	LK.A..EYV.	..L..SL.CQ	.K.TPS.QA.	..ASE...VS	
rat A	.....S.	.K.....KE.	LK.A..EYI.	..L..SL.CQ	.K.TPS.Q..	..ASE...IS	
<i>DrALD-A</i>	.....S.	.K.....KE.	GK.C..E.I.	..LN.S..CV	.K..SS.DK.	..AGE...V.	
<b>ALD-A/C</b>	.....S.	.K.....KE.	LK.A..E.I.	..E..GL..Q	.K..PS.?.	..ASE...V.	
human C	.....S.	.N..R.QRD.	AG.AT.E.I.	..EV.GL..Q	.K.EGS.ED.	G.AA...YI.	
rat C	.....S.	.S..R.QRD.	AG.AT.E.I.	..EM.GL..Q	.K.EGS.-D.	G.AA...YV.	
goldfish C	.....S.	.S..R.VKE.	EK.AT.E.L.	..E..GL..Q	.K..SS.-MD	GSAG...YV.	
pufferfish C	.....S.	.N..K.ELS.	EK.AT.E.I.	..E..GL..L	.K.ECS.-TA	G.AA...YVE	
<i>DrALD-C</i>	.....S.	.S..R.AKS.	EK.AT.E.I.	..E..GL..Q	.K..SS.-TC	G.AG...YV.	
<b>ALD-C</b>	.....S.	.N..R..KE.	AK.AT.E.I.	..E..GL..Q	.K..SS.?.	G.AA...YV.	
human B	CYTY 364	pI=8.2					
rat B	S...	8.4					
sea bream B	S.V.	8.1					
<i>DrALD-B</i>	S...	8.2					
Human A	NHA.	8.1					
rat A	NHA.	8.1					
<i>DrALD-A</i>	NHA.	8.2					
<b>ALD-A/C</b>	NHA.	7.9					
human C	NHA.	6.4					
rat C	NHA.	6.7					
goldfish C	NHA.	6.4					
pufferfish C	NHA.	6.2					
<i>DrALD-C</i>	NHA.	6.2					
<b>ALD-C</b>	NHA.	8.1					

Fig. 1. Continued.

To confirm that the three zebrafish cDNAs had the characteristic tissue-specific expression patterns of aldolase isozymes reported in other species, we designed oligonucleotide primers specific for each gene and used RT-PCR amplification to assay for the presence of specific mRNA across a variety of zebrafish tissues [data not shown; see Merritt and Quattro (2001) for a more detailed description of this approach]. Primers specific for *DrALD-A* amplified a product from every tissue examined (eye, brain, ovary, liver, gill, gut, and muscle), whereas specific primers for *DrALD-B* amplified product from every tissue except brain. Primers specific for *DrALD-C* amplified a product only from eye, brain, and

ovary. PCR products were sequenced to verify their identity as *DrALD-A*, *DrALD-B*, and *DrALD-C*. The presence of *DrALD-A* and *DrALD-B* message across all tissues examined might seem surprising given that these are predominantly muscle and liver isozymes (respectively). However, while histochemical staining indicates that both isozymes are expressed at highest levels in their respective tissues, they are both expressed at lower levels across a variety of tissues (Morizot and Schmidt 1990). Given this, the pattern of aldolase mRNA expression matches the pattern of protein expression reported for other species (e.g., Lebherz and Rutter 1969; Horecker et al. 1972). Further, locus identi-



**Table 1.** Number of amino acid substitutions that alter or conserve a given amino acid property along branches of the aldolase phylogeny<sup>a</sup>

	Reconstructed changes along branch(es)		
Property	<i>A/C–C</i>	Tree	<i>p</i>
<b>Charge</b>			
Conserve	8 (5)	372 (329)	0.44 (0.39)
Alter	4 (3)	142 (124)	
<b>Polarity</b>			
Conserve	8 (6)	323 (301)	0.53 (0.47)
Alter	4 (2)	190 (152)	
<b>Size</b>			
Conserve	7 (5)	332 (306)	0.43 (0.51)
Alter	5 (3)	181 (147)	

<sup>a</sup> Branches are as defined in Fig. 2. “Tree” refers to summed changes along all branches excluding *A/C–C*. *p* is the result of a Fisher’s exact test that the observed number of changes within properties is homogeneous across branch designations. Results of a similar analysis excluding four ambiguously reconstructed amino acid positions (see Results) are shown in parentheses. See Methods for details.

and size were included as possible contrasts to any patterns we might have observed in charge changes.

There are 12 amino acid changes along the *A/C–C* branch of the aldolase gene tree (Fig. 1, Table 1). Four of the 12 amino acid changes result in a change in amino acid charge. However, in direct contrast to the pattern of amino acid substitution along the analogous branch in the evolution of teleost neural TPI, there are no changes along the *A/C–C* branch to negative amino acids. Two changes are from positively charged amino acids to neutral amino acids (histidine to proline and lysine to asparagine). One change is from a negatively charged amino acid to a neutral amino acid (glutamate to glutamine). One change is from a neutral amino acid to a positively charged amino acid (glycine to arginine). Eight changes do not result in changes in charge. There are 514 amino acid changes across the rest of the aldolase gene tree; 142 alter charge, whereas 372 maintain amino acid charge. The ratios of no charge change to charge change, 8/4 and 372/142, are not significantly different (Fisher’s exact test, *p* = 0.44; Table 1). Similar results were obtained when amino acids were grouped by polarity or size (Table 1). The pattern of amino acid substitution, and concomitant charge change, along the *A/C–C* branch of the aldolase gene tree does not significantly differ from that across the rest of the aldolase gene tree.

The inferred amino acids at some amino acid positions were weakly supported. To avoid the possibility of incorrectly inferred positions masking any difference in the patterns of amino acid substitutions between the *A/C–C* branch and the rest of the tree similar statistical tests were conducted after ambiguous sites had been excluded. Sites were deemed ambiguous if the most likely amino acid was less than

twice as probable as the second most likely amino acid (e.g., Zhang et al. 1998). Using this criterion, 14 positions of the *A/C* or *C* sequences (amino acids 10, 45, 58, 65, 70, 92, 95, 97, 156, 293, 319, 333, 345, 353; Fig. 1) could be construed as ambiguous and were excluded from further analyses. With these ancestral sites excluded, there are five amino acid changes along the *A/C–C* branch that maintain amino acid charge and three that result in change to a more positively charged amino acid. As in the previous analysis, no amino acid changes result in a negatively charged amino acid. Across the rest of the gene tree there are 329 amino acid changes that maintain charge and 124 that result in a charge change (Table 1). The ratio 5/3 does not significantly differ from 329/124 (Fisher’s exact test, *p* = 0.39; Table 1). Again, similar results were obtained when amino acids were grouped by polarity or size (Table 1). The pattern of amino acid change along the *A/C–C* branch, after removal of the ambiguous sites, was still not significantly different from that observed across the rest of the tree. While the aldolase C isozymes of modern vertebrates are more negatively charged than their paralogous aldolase counterparts, we have no evidence that this results from the accumulation of negatively charged amino acids directly following the duplication that led to the ancestral *Ald-C* gene.

Discussion

Electrophoretic separation of proteins from a variety of vertebrate tissues revealed a correlation between neural expression and a net negative charge (e.g., Moore and McGregor 1965; Moore 1973). The generality of this pattern across gene families suggested that this negative charge might be an adaptation to the neural environment (Fisher et al. 1980). Our study of the TPI gene family (Merritt and Quattro 2001) indicated that the negative charge of neural TPI (TPI-A) was the product of positive selection. TPI-A, however, is found only in teleost fish and the applicability of our findings to the evolution of other gene families is therefore limited. To investigate the generality of our findings we have investigated the evolution of the aldolase gene family, which includes a neural isozyme (aldolase C) in all gnathostome vertebrates.

We sequenced all three aldolase isozymes from a single teleost species, the zebrafish. Phylogenetic analysis of the three zebrafish aldolase sequences, and other aldolase sequences from GenBank, supports earlier conclusions (e.g., Kuraku et al. 1999; Berardini et al. 1997) that the *Aldolase-A*, *-B*, and *-C* genes of gnathostome vertebrates form three orthologous groups. The well-supported tree suggests two gene duplication events following the separation of the agnathan and gnathostome vertebrates but predating the separation of the teleost and tetrapod lineages.

The predicted isoelectric points of the zebrafish aldolase isozymes (Fig. 1) indicate that the neural isozyme is more negatively charged than either of the other zebrafish aldolase isozymes and that the amino acid sequence alone can account for differences in charge across the three isozymes. The fact that the zebrafish neural aldolase is more negatively charged than either of the other zebrafish aldolase isozymes is consistent with the correlation of charge and neural expression observed across aldolase isozymes in other vertebrate taxa (e.g., human and rat) and across other vertebrate gene families (e.g., teleost TPI and LDH). From this we conclude that the correlation within gene families of net negative charge and neural expression reported in teleost fish (e.g., Fisher et al. 1980) is present across jawed vertebrates as a whole.

In contrast to our earlier study of the TPI gene family (Merritt and Quattro 2001), we find no evidence for positive selection following the duplication that gave rise to the neural aldolase. The rate of synonymous substitution exceeded the rate of non-synonymous substitution across the branch between the duplication and the ancestral aldolase C on the aldolase gene tree (the *A/C-C* branch; Fig. 2), suggesting purifying, not positive, selection. Similarly, comparison of the inferred ancestral amino acid sequences across the *A/C-C* branch indicates no bias in amino acid substitution when amino acids are classified by charge, polarity, or size. Like the neural TPI, the neural aldolase isozyme evolved a net negative charge, but unlike the TPI case, reconstructed aldolase sequences (nucleotide and amino acid) do not suggest an unusual pattern of substitution leading to the phenotype of negative charge.

Examination of the five modern aldolase C proteins (Fig. 1) provides a possible explanation for the lack of apparent evidence for selection. While all five modern aldolase C sequences have negative isoelectric points, they share only a single negatively charged amino acid unique to the aldolase C clade (amino acid 333; Fig. 1). There are three negative amino acids unique to teleost aldolase C proteins (amino acids 71, 161, and 321) and one negative amino acid unique to the tetrapod aldolase C proteins (amino acid 349). The characteristic of a net negative charge shared across the aldolase C sequences of teleosts and tetrapods is, therefore, predominantly a product of different amino acids in either group. The inferred ancestral aldolase C has a predicted isoelectric point of 8.1, even though no modern aldolase C protein has a predicted isoelectric point higher than 6.7 (Fig. 1), reflecting this lack of shared negatively charged amino acids. Ancestral reconstruction using other methods, including parsimony (data not shown), also failed to predict an ancestral aldolase C with a net negative charge. The lack of evidence for an excess of nonsynonymous substitution, or of a bias in amino acid substitutions,

directly following the gene duplication that gave rise to *Ald-C*, likely reflects the lack of a common set of negatively charged amino acids across vertebrate aldolase C sequences.

The small number of shared negatively charged amino acids (one) suggests that the negative phenotype common to all vertebrate aldolase C proteins might have evolved separately in the tetrapod and teleost lineages. Aldolase C may, then, represent an interesting case of convergence on negative charge among taxa within a single gene family, but this appears unlikely for several reasons. Analysis of the TPI gene family indicates that the negative charge of the neural TPI evolved quickly after the duplication event unique to bony fishes (Merritt and Quattro 2001). Given the timing of the duplication that gave rise to the *Ald-C* gene, if similar selective forces were acting on the aldolase C protein, it seems likely that the ancestral aldolase C protein would have acquired a negative charge before the divergence of tetrapods and teleosts. In this case, the different sets of negatively charged amino acids in teleost and tetrapod aldolase C would represent divergence, not convergence, since the time of their last common ancestor. The pattern of synonymous and nonsynonymous changes indicates only purifying selection across all branches in question (Fig. 2), and so does not help in distinguishing between these two possibilities. Our statistical analysis and reconstruction argue against a negatively charged ancestral aldolase C. However, given the small number of amino acids involved in the negative charge, it seems possible that this analysis is simply not sensitive enough to track accurately the evolution of the negative charge phenotype.

The available aldolase sequences provide some circumstantial evidence to distinguish between convergence and divergence. The negative amino acids of the aldolase C proteins vary to some degree within both teleost fish and tetrapods (e.g., amino acid positions 354 and 360; Fig. 1). This variation suggests that the negatively charged amino acids of neural aldolase isozymes are not fixed but are free to vary as long as the overall net negative charge of the protein is maintained. In other words, variation in the negatively charged aldolase C amino acids suggests that the selective pressure is for a net negative charge, not for a particular negatively charged amino acid at a particular position within the protein. A similar situation is present in the teleost neural TPI isozyme (Merritt and Quattro 2001); the overall net negative charge is maintained, but in the two teleost species examined the exact amino acids responsible for the negative charge vary to a small extent. In the case of the teleost neural TPI isozyme, the teleost species are separated by only approximately 100 million years (Patterson 1993) and variation already exists. In the case of the vertebrate neural aldolase, the duplication



that gave rise to the neural isozyme appears to have occurred sometime between the separation of the agnathan and gnathostome vertebrates approximately 500 MYA (Dayhoff 1978) and the separation of fish and tetrapods approximately 400 MYA (Dickerson 1971). Given the length of time separating the modern aldolase sequences, if a negative charge was the ancestral condition, it is not surprising that the amino acids involved in the negative phenotype of the modern neural isozymes vary. The information currently available does not allow us to distinguish between the hypotheses of parallel evolution of a negative charge and divergence from a common (negatively charged) ancestral condition. Examination of aldolase C proteins, their charge, and any unique negatively charged amino acids from taxa closer to the separation of the two lineages might shed light on this question. However, it might be that the period of interest is simply too long ago and that the necessary information has been obscured by multiple substitutions.

The biological significance of the negative charge of neural isozymes is elusive. In some gene families, biochemical differences have been demonstrated between the neural isozymes and their nonneural counterparts. Teleost LDH-C, for example, has kinetic and thermal tolerance characteristics distinct from those of LDH-B (Whitt 1970; Shaklee et al. 1973), the LDH isozyme to which it is most closely related (Quattro et al. 1993). The neural creatine kinase also has kinetic properties distinct from those of the nonneural isozyme (Eppenberger et al. 1967). In mammals, all three aldolase isozymes have distinct substrate preferences (Kusakabe et al. 1994). Conversely, some gene families with negative neural isozymes have no demonstrable biochemical differences between tissue-specific isozymes [e.g., the enolase gene family (Marangos and Schmechel 1987)]. Detection of biochemical differences between isozymes *in vitro* does not equate to demonstration of meaningful biological differences *in vivo*. Similarly, failure to detect differences *in vitro* does not necessarily mean that no differences exist *in vivo*. The strong evidence for directional selection in the evolution of the teleost neural TPI indicates that there is a selective advantage to the net negative charge of this neural isozyme (Merritt and Quattro 2001). By inference, this evidence from the TPI gene family and the correlation of negative charge and neural expression across other gene families imply a biological significance for the negatively charged isozymes as a group.

The number of examples of protein families that include a negatively charged neural isozyme further suggests that the selective advantage involved in the evolution of the net negative charge is a general phenomenon not restricted to a particular type of biochemical reaction or pathway. Importantly, the

neural isozymes are not expressed at the exclusion of the other, more generally expressed, isozymes, e.g., both TPI-B and aldolase A are expressed in neural tissues along with their exclusively neural paralogs. This suggests that a negative charge is not required for function in the neural environment. By extension, this further suggests that the negative charge might be in response to selection for a novel function, unique to the neural environment. We have suggested that one such general selective pressure could be the accumulation of negatively charged proteins necessary for maintenance of the resting membrane potential (Merritt and Quattro 2001). Other such general requirements of neural tissues undoubtedly exist. Investigation of other protein families that include negatively charged neural isozymes and, perhaps even more importantly, protein families that do not show this correlation might help address the question of the biological significance of the negative charge.

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