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Evidence that mitochondrial isozymes are genetically less variable than cytoplasmic isozymes

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Summary

It has been proposed that isoenzymes functioning within cell organelles (chloroplasts, mitochondria) are genetically less variable than their cytoplasmic counterparts, as a result either of constraints imposed by the need to cross organelle membranes or from the different and specialized nature of organelle metabolism. However, some recent findings concerning chloroplast and cytoplasmic isozyme variability are not consistent with this thesis. We have analyzed a number of surveys of electrophoretically detectable enzyme variation in vertebrates, and show that for each of the four tested enzymes (malate dehydrogenase, isocitrate dehydrogenase, malic enzyme, and aspartate aminotransferase), the mitochondrial isozymes are less variable than their corresponding cytosplasmic forms. The mean heterozygosities across the four enzymes are 0·083 and 0·038 for the cytoplasmic and mitochondrial forms respectively. We conclude that mitochondrial isozymes are indeed subject to greater constraints than cytoplasmic forms and have fewer sites able to accept neutral or slightly deleterious mutations. It is also noted that of the enzymes analyzed, that with the smallest subunit molecular weight (MDH) has the least variable cytoplasmic and mitochondrial isozymes, whereas the enzyme with the largest subunits (ME) has the most variable isozymes.

1. Introduction

Different enzymes, when assayed by electrophoresis, show different average levels of heterozygosity, and many studies have attempted to relate this variation to structural or catalytic properties of the enzymes, for example subunit number (Harris, Hopkinson & Edwards, 1977; Ward, 1977), subunit molecular weight (Eanes & Koehn, 1978; Koehn & Eanes, 1978; Nei, Fuerst & Chakraborty, 1978; Ward, 1978; Brown & Langley, 1978), substrate type (Gillespie & Kojima, 1968; Kojima, Gillespie & Tobari, 1970), and regulatory function (Johnson, 1974).

It is generally true that when an enzyme is present in more than one subcellular compartment, then distinct isozymes characterize the individual compartments. For example, malate dehydrogenase (MDH, EC 1.1.1.37), malic enzyme (ME, EC 1.1.1.42), isocitrate dehydrogenase (IDH, EC 1.1.1.42), superoxide dismutase (SOD, EC 1.15.1.1), aspartate aminotransferase (AAT, EC 2.6.1.1) and aconitase (ACO, EC 4.2.1.3) exist as both mitochondrial and cytoplasmic isozymes, and in each case it has been shown that the two isozymes are encoded by separate nuclear genes. Fumarase provides an exception to this generalization, since here both cytoplasmic and mitochondrial forms are encoded by a single nuclear locus. So far, rather little attention has been paid to the possible effects of differences in subcellular localization on the degree of genetic variability, although in plants Gottlieb & Weeden (1981) have shown that the locus specifying the chloroplast isozyme of phosphoglucose isomerase is substantially less variable than that encoding the cytoplasmic isozyme. Gottlieb (1982) thus proposed that subcellular location may have a significant influence on levels of 'permissible' variation of coding genes. However, subsequently two other enzymes in plants, triose phosphate isomerase and glyceraldehyde-3-phosphate dehydrogenase (G3PDH), were shown to be more variable in plastid form (Cerff & Kloppstech, 1982; Pichersky & Gottlieb, 1983).

Selander (1976; Selander & Johnson, 1973) suggested, although without statistical tests, that the mitochondrial isozymes of IDH and AAT in vertebrates were less variable than their cytoplasmic counterparts, but that there were no such differences for MDH. Interestingly, van Heyningen, Craig &

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Bodmer (1973), in a study of somatic cell hybrids, noted that human and mouse mitochondrial isozymes of MDH, AAT and IDH were difficult or impossible to separate electrophoretically, a problem that did not arise with the corresponding cytoplasmic forms. They suggested possible charge conservation among the mitochondrial isozymes.

The amount of data now available is more extensive than that of ten or more years ago, and thus a re-investigation of this topic seems appropriate. The null hypothesis we test is that there are no differences in the degree of genetic variability between cytoplasmic and mitochondrial isozymes. In some of our tests, because data for each type of isozyme can be obtained from single species, observed differences in heterozygosity between mitochondrial and cytoplasmic isozymes cannot be confounded with effects due to the use of different sets of enzymes. This is not true of tests made previously which assessed the effects of, for example, subunit size and number (see above).

2. Materials and methods

Data were collected from published surveys of vertebrate species that screened by electrophoresis fifteen or more loci from fifteen or more individuals. Such surveys represent attempts to obtain estimates of levels of genetic variation at loci not chosen for their tendency to polymorphism. The present paper is restricted to consideration of a small subset of that database, namely MDH (malate dehydrogenase, EC 1.1.1.37), ME (malic enzyme, EC 1.1.1.40), IDH (isocitrate dehydrogenase, EC 1.1.1.42) and AAT (aspartate aminotransferase, EC 2.6.1.1), when likely subcellular locations of the enzymes were given in the source paper. Note that unfortunately in the majority of publications this information is not presented. This is particularly true for SOD (superoxide dismutase, EC 1.15.1.1), and for this enzyme and the rather infrequently screened enzyme ACO (aconitase, EC 4.1.2.3), too few data were available for analysis. For each locus the average (Hardy–Weinberg expected) heterozygosity was calculated from the allele frequencies. Where data from more than one population per species were presented, then the unweighted mean allele frequencies across populations were used as the basis of the heterozygosity calculations. Most of the source papers are given in Skibinski & Ward (1981) and Ward & Skibinski (1985).

Individual locus heterozygosity values were non-normally distributed, and for each of the four enzymes analysed, the distributions of the cytoplasmic and mitochondrial locus heterozygosities were compared using two non-parametric tests. Firstly, the Mann-Whitney *U* test was used to compare the heterozygosity distributions of all mitochondrial loci for a particular enzyme against those of the cytoplasmic loci [data set (i)]. However, since the set of species used to provide mitochondrial data was not identical to the set providing cytoplasmic data (for example, only the cytoplasmic isozyme of MDH was scored in some surveys), there was the possibility that differences in heterozygosity distributions may have reflected differences in species composition rather than real differences attributable to subcellular location of the isozymes. In order to guard against this possibility, a second set of data (ii) was analyzed. This comprised only those species (n) scored for both the cytoplasmic and mitochondrial isozymes of the enzyme under investigation. Thus n-paired comparisons were available and were assessed using Wilcoxon's matched-pairs test. In a number of cases isozyme duplication has occurred so that two (or, occasionally three or four) loci code for the particular isozyme. In such cases, each locus value contributed to data set (i) and was used in the Mann-Whitney test, but in data set (ii) the mean heterozygosity of the duplicated loci was used as the cytoplasmic or mitochondrial value and analysed by the Wilcoxon test.

Numbers of polymorphic and monomorphic loci were compared between enzymes and locations using a three-way G test employing log-linear models (Sokal & Rohlf, 1981). Overall mean heterozygosities per cytoplasmic or mitochondrial locus were calculated, together with their standard errors. An analysis of variance (ANOVA) was performed on these mean values, and the variance components estimated.

3. Results

Table 1 summarizes the data. The Mann–Whitney test indicates that for three of the enzymes, MDH, IDH and AAT, the cytoplasmic isozymes are significantly more variable than the mitochondrial forms. The fourth enzyme, ME, much less well sampled than the other three, displays the same trend although no statistical significance can be attached to the result. For each enzyme, the mitochondrial isozyme has a higher percentage of monomorphic loci than the cytoplasmic form. The unweighted mean heterozygosity per locus across the four enzymes is 0·0825 and 0·0375 for the cytoplasmic and mitochondrial isozymes respectively.

The Wilcoxon test shows that for two of the four enzymes, IDH and AAT, the cytoplasmic isozymes are significantly more variable than the mitochondrial forms. For MDH and ME the differences are non-significant but the cytoplasmic forms have the higher mean heterozygosity. Again, monomorphism is more frequent in mitochondrial isozymes. The unweighted mean heterozygosity per locus across the four enzymes is now 0·0803 and 0·0420 for the cytoplasmic and mitochondrial isozymes respectively. On average, and looking at the results of both of the tests, cytoplasmic isozymes appear to be about twice as heterozygous as mitochondrial forms.

An analysis of the numbers of monomorphic and

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Table 1. *Average heterozygosities per locus and percent monomorphism for cytoplasmic (cyt) and mitochondrial (mit) isozymes of MDH, ME, IDH and AAT*

Locus	Heterozygosity/locus ± S.E.		Loci % Mono.	Loci Total	No. loci screened per phylum Fish	Amph.	Bird	Rept.	Mamm.
MDH	(i) Cyt	0·037 ± 0·010	79·7	123	78	3	9	9	24
	Mit	0·014 ± 0·006	90·4	73	30	3	9	9	22
	Mann–Whitney test: $Z = 1·96$, $P = 0·05$								
	(ii) Cyt	0·021 ± 0·008	77·6	67	25	3	9	9	21
	Mit	0·015 ± 0·007	89·6	67	25	3	9	9	21
	Wilcoxon matched-pairs test: $T = 63$, $n = 19$, $P > 0·05$								
ME	(i) Cyt	0·150 ± 0·040	42·9	14	2	2	—	—	10
	Mit	0·096 ± 0·026	78·6	14	3	2	—	—	9
	Mann–Whitney test: $U = 71$, $P > 0·05$								
	(ii) Cyt	0·141 ± 0·060	66·0	10	2	2	—	—	6
	Mit	0·109 ± 0·058	70·0	10	2	2	—	—	6
	Wilcoxon matched-pairs test: $T = 8$, $n = 6$, $P > 0·05$								
IDH	(i) Cyt	0·075 ± 0·021	61·0	41	4	3	—	9	25
	Mit	0·018 ± 0·008	79·0	38	7	2	—	9	20
	Mann–Whitney test: $Z = 1·99$, $P = 0·05 > P > 0·02$								
	(ii) Cyt	0·090 ± 0·024	55·9	34	3	2	—	9	20
	Mit	0·020 ± 0·009	76·5	34	3	2	—	9	20
	Wilcoxon matched-pairs test: $T = 29$, $n = 19$, $0·01 > P > 0·001$								
AAT	(i) Cyt	0·068 ± 0·016	63·0	81	30	3	9	9	30
	Mit	0·022 ± 0·010	87·2	78	33	3	9	3	30
	Mann–Whitney test: $Z = 3·57$, $P < 0·001$								
	(ii) Cyt	0·069 ± 0·017	62·5	72	27	3	9	3	30
	Mit	0·024 ± 0·011	86·1	72	27	3	9	3	30
	Wilcoxon matched-pairs test: $T = 134$, $n = 34$, $0·01 > P > 0·001$								

Note: data sets (i) and (ii) are explained in the text. Probabilities are from two-tailed tests.

Table 2. *Analysis of three-way tables using log-linear models*

	Variability (V)							
	Data set (i) Numbers of loci Location (L)				Data set (ii) Numbers of loci Location (L)			
	Monomorphic		Polymorphic		Monomorphic		Polymorphic	
Enzyme (E)	Cyt	Mit	Cyt	Mit	Cyt	Mit	Cyt	Mit
MDH	98	66	25	7	52	60	15	7
ME	6	11	8	3	6	7	4	3
IDH	25	30	16	8	19	26	15	8
AAT	51	68	30	10	45	62	27	10

Initial model: $\ln \hat{f}_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk}$ where $\alpha \equiv V$, $\beta \equiv L$, $\gamma \equiv E$.

Component	Data set (i) G	D.F.	P	Data set (ii) G	D.F.	P
3-factor interaction:						
Test $\alpha\beta\gamma = 0$	1·221	3	n.s.	0·919	3	n.s.
2-factor interactions:						
(*a*) Test $\alpha\beta_{ij} = 0$	23·577	4	**	17·563	4	**
After dropping $\alpha\beta$	22·643	1	***	16·913	1	***
(*b*) Test $\alpha\gamma_{ik} = 0$	16·817	6	**	10·575	6	n.s.
After dropping $\alpha\gamma$	15·758	3	**	9·781	3	*
(*c*) Test $\beta\gamma_{jk} = 0$	11·433	6	n.s.	1·359	6	n.s.
After dropping $\beta\gamma$	10·315	3	*	0·421	3	n.s.

Final model: $\ln \hat{f}_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\beta_{ij} + \alpha\gamma_{ik}$[$+ \beta\gamma_{jk}$ in data set (i)]

Probability levels: * $0·05 > P > 0·01$; ** $0·01 > P > 0·001$; *** $P < 0·001$.
Note: Williams' correction used as and when recommended by Sokal & Rohlf (1981).

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Table 3. *Analysis of variance of heterozygosity data set (ii) from Table 1*

	Mean heterozygosity per locus		
	Cyt	Mit	Mean
MDH	0·021	0·015	0·018
ME	0·141	0·109	0·125
IDH	0·090	0·020	0·055
AAT	0·069	0·024	0·047
Mean	0·080	0·042	0·061

ANOVA table

Source of variation	D.F.	SS	MS	*F*	*P*
Enzymes (*E*)	3	0·0123824	0·0041275	11·612	0·037
Location (*L*)	1	0·0029262	0·0029262	8·232	0·064
Interaction ($E \times L$)	3	0·0010664	0·0003555		
Total	7	0·0163749			

Added variance components (Model II ANOVA assumed)

Factor	Variance	Total (%)
Enzymes (*E*)	0·0018860	65·4
Location (*L*)	0·0006427	22·3
Interaction ($E \times L$)	0·0003555	12·3

polymorphic loci in both data sets (i) and (ii) of Table 1 has been carried out in a three way classification using the method of log-linear models as described in Sokal & Rohlf (1981). The three-way table (Table 2) is *V* (variation, 2 levels, monomorphic and polymorphic) × *L* (location, 2 levels, cytoplasmic and mitochondrial) × *E* (enzyme, four levels, MDH, ME, IDH, and AAT). This analysis indicates that the three-way interaction *VLE* is not significant, and thus the degree of association between *V* and *L* does not differ for the different enzymes. However, there are significant associations between *V* and *L* given the level of *E* ($\alpha\beta = 0$) and between *V* and *E* given the level of *L* ($\alpha\gamma = 0$). These results indicate that there is significant variation in the relative proportions of monomorphic and polymorphic loci between locations and between enzymes, and thus corroborate the results of the non-parametric tests outlined earlier. In data set (i), there is a significant association between *L* and *E* given the level of *V*. This arises from the excess of cytoplasmic loci over mitochondrial loci for MDH and the approximate equalities of these two parameters for the other enzymes. Data set (ii) was constructed in such a way that this imbalance was not present.

An analysis of variance of the mean heterozygosity per locus of data set (ii) is provided in Table 3. An analysis of data set (i) gave similar results. Table 3 shows that variation attributable to enzymes is significant at the 5% level, with variation attributable to location bordering on statistical significance. More interestingly, the total variation can be partitioned between enzymes and between locations. This shows that the added variance due to location (22·3%) is about one third that due to enzyme (65·4%). This, taken together with the earlier analyses, indicates that subcellular location is an important contributor to variation in allozyme heterozygosity.

It is also interesting that the order of variability within the cytoplasmic and within the mitochondrial forms is MDH < IDH/AAT < ME. This order reflects differences in subunit molecular weight which range from 35×10^3 for MDH through 46 and 48×10^3 (AAT and IDH respectively) to 60×10^3 for ME (molecular weights are those of human enzymes, Hopkinson, Edwards & Harris, 1976; see also Dixon & Webb, 1979). There is very little difference in subunit molecular weight between the cytoplasmic and mitochondrial forms of each of these enzymes, and their quaternary structure is conserved (MDH, IDH and AAT being dimers, ME a tetramer) (Hopkinson *et al.* 1976; Dixon & Webb, 1979).

4. Discussion

The hypothesis that isozymes encoded by nuclear genes and functioning within cell organelles should be less variable than cytoplasmic enzymes (Gottlieb & Weeden, 1981; Gottlieb, 1982) is supported by our results. Vertebrate mitochondrial MDH, IDH and AAT (and probably also ME) are all less variable than their cytoplasmic counterparts.

Ironically, this hypothesis, first formulated following the observation that in a large number of plant genera chloroplast phosphoglucose isomerase was less variable than the cytoplasmic enzyme (Gottlieb & Weeden, 1981), was later contradicted by studies of two additional chloroplast/cytoplasmic pairs of isozymes. Plastid triose phosphate isomerase (in members of a single genus, *Clarkia*) and G3PDH (in a variety of

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genera) were both shown to be more variable than the corresponding cytoplasmic enzymes (Pichersky & Gottlieb, 1983; Cerff & Kloppstech, 1982). However, at least with respect to G3PDH, the two isozymes, although possibly descendants of a common ancestor (Cerff & Chambers, 1979), and unlike the pairs of cytoplasmic and mitochondrial isozymes we have analysed, differ in a number of important structural and functional aspects. For example, the cytoplasmic form of G3PDH is NAD-specific, the plastid form NADP-specific. Furthermore, the cytoplasmic form is a homotetramer, whereas the plastid form is generally made of two distinct isozymes, A4 and A2B2, although there is interspecies heterogeneity in subunit number (Cerff, 1982). Hence the G3PDH isozymes seem to have differentiated far more than is typical for cytoplasmic/organelle pairs or enzymes. Indeed, the two isozymes are sufficiently distinct as to warrant different EC numbers (1.2.1.12 and 1.2.1.13 respectively). This is not the case for any of the other isozyme pairs considered here. Thus if we exclude G3PDH from consideration, five enzymes (MDH, IDH, ME, AAT and PGI) are less variable in organelle form, and one enzyme (TPI, so far only tested in a single genus) more variable. We conclude that mitochondrial isozymes are less variable than cytoplasmic isozymes, but that more data need to be gathered to show whether plastid isozymes are less variable than their cytoplasmic counterparts.

So far, with the exception of aspartate aminotransferase, rather few comparisons have been made on the amino acid compositions of vertebrate cytoplasmic and mitochondrial isozymes. However, results from amino acid sequencing indicate that the degree of sequence identity between pig and chicken mitochondrial AAT (86%) is similar to that between cytoplasmic AAT from the same two species (83%) (Graf-Hausner, Wilson & Christen, 1983; Doonan, Barra & Bossa, 1984). Hence mitochondrial and cytoplasmic AAT appear to have been evolving at approximately equal rates. Yet immunological distances between the cytoplasmic AAT of pig and other species (other mammals, birds, reptiles and amphibians) are about twice as great as for the mitochondrial isozymes (Sonderegger & Christen, 1978). The apparent conflict in the results of these two methodologies was largly resolved when Graf-Hausner *et al.* (1983) identified 36 residues (9% of total) that were specificially conserved in pig/chicken mitochondrial AAT in comparison with only 10 (2%) conserved in the cytoplasmic isozymes. These specifically conserved sites were located in the surface regions, away from the active site and regions of intersubunit contact. Some of these surface regions are antigenic. Graf-Hausner *et al.* conclude that there are indeed evolutionary constraints imposed upon mitochondrial isozymes that are absent from cytoplasmic isozymes. Note that it is principally surface-site variation which is picked up by the electrophoretic methods used by the authors of the papers we surveyed, and hence our results support the contention that mitochondrial isozymes have evolutionarily conserved surface regions.

Our data indicate that the average heterozygosity of cytoplasmic isozymes is about twice that of mitochondrial isozymes. Consideration of both the stepwise neutral model of evolution (Ohta & Kimura, 1973) and that invoking very slightly deleterious mutations (Ohta, 1976) to explain the observed levels of variation, shows that whether the mutations contributing to heterozygosity are neutral or slightly deleterious, a doubling of the mutation rate for these kinds of mutations will lead to an approximate doubling in heterozygosity at these low heterozygosity values. In both situations highly deleterious mutations, those that contribute little to heterozygosity, would be more common in mitochondrial than cytoplasmic isozymes.

What is it that constrains variability in enzymes functioning in mitochondria? For the enzymes we studied, molecular weights and quaternary structures of the two forms are in each case very similar, and cannot be the cause of the observed differences. There are two possibilities: first, that the requirement for cross-membrane transport into organelles constrains variability, second, that the internal environment of organelles selects for isozymes with different properties and hence different constraints from the cytoplasmic forms. The mitochondrial enzymes considered here are located in the mitochondrial matrix (Dixon & Webb, 1979), and hence have to pass across both inner and outer membranes. Most mitochondrial (and plastid) enzymes synthesized on cytoplasmic ribosomes are synthesized with a transient amino-terminal leader peptide, which, possibly in combination with a 40 kDa import protein, targets the enzyme to specific receptors on the outer membrane of the mitochondrion (Wickner & Lodish, 1985). It has been proposed that the targetting leader peptide must fold into an 'import-competent' structure and must be exposed on the surface of the precursor molecule (Hurt & Schatz, 1987). Movement of the enzyme across the membranes then takes place, and in the matrix the leader sequence is removed by a protease (Wickner & Lodish, 1985). It seems not unreasonable to propose that the complexities of the molecular interactions required to transport proteins across membranes constrain amino acid variability or organelle isozymes in ways not encountered by cytoplasmic forms, athough it should be pointed out that the targetting leader sequence apparently folds independently of the enzyme proper (Hurt & van Loon, 1986; Eilers & Schatz, 1986) and thus itself may not in any major way constrain variability of the mature enzyme. However, it has been proposed that the surface conserved regions of mitochondrial AAT, referred to above, are also involved in the translocation of the enzyme into mitochondria (Doonan *et al.* 1984). The removal of such constraints in cytoplasmic

isozymes means that for these isozymes a greater proportion of new mutations is likely to be neutral or slightly deleterious than is the case for mitochondrial isozymes: hence cytoplasmic isozymes may be predicted to be more variable than mitochondrial forms. Consistent with such an explanation are the findings that water soluble proteins in mice are substantially more variable than membrane-bound proteins (Klose & Feller, 1981), and that structural proteins are more highly conserved than soluble enzymes (Kimura, 1983).

The second possibility, that subcellular environment *per se* has an effect on enzyme variability, is more difficult to assess. Certainly, the environments are quite different, but it is hard to make an *a priori* prediction of the effect that this will have on enzyme variability. For example, the pH of the mitochondrial matrix is around 8 whereas the cytoplasm has a pH of around 7 (Hinkle & McCarty, 1978). Furthermore, catalytic behaviours of the two isozymes may differ in the two compartments. Mitochondrial MDH is primarily concerned with the forward reaction (in the citric acid cycle) whereas cytoplasmic MDH may be more concerned with the reverse reaction (perhaps in lipogenesis) (Dixon & Webb, 1979).

The fact that of the four enzymes considered here, that with the smallest subunit molecular weight (MDH) is the least variable in both forms and that with the largest weight (ME) the most variable supports the contention that subunit molecular weight is one of the important determining factors in determining levels of genetic variability (Koehn & Eanes, 1978; Nei *et al.* 1978; Ward, 1978). However, neither this factor nor level of quaternary structure (Harris *et al.* 1977; Ward, 1977) explains why mitochondrial enzymes are less heterozygous than cytoplasmic forms. Constraints imposed by cross-membrane transport or functional differentiation may be responsible for this reduced variability. It seems that subcellular location must be added to the list of factors, both structural and functional, which affect levels of enzyme variability.

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