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Genetics

Expression of alkaline phosphatase loci in mammalian tissues

(inhibitors/thermostability/evolution)

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ABSTRACT **Alkaline phosphatases [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] have been examined in liver, bone, kidney, intestine, and placenta from nine mammalian species by quantitative inhibition and thermostability studies and compared with alkaline phosphatases in the corresponding human tissues. In humans, three kinds of alkaline phosphatase can be sharply differentiated by these methods, one occurring in liver, bone, and kidney, one in intestine, and one in placenta. They are evidently determined by separate gene loci. In the mammals only two sorts of alkaline phosphatase were found: one, which occurs in liver, bone, kidney, and also placenta, corresponds to the human liver/bone/kidney enzyme and the other corresponds to the human intestinal enzyme. The findings support our earlier proposal that the expression of a distinctive type of alkaline phosphatase in human placenta is the consequence of a late evolutionary event which occurred subsequent to the divergence of the evolutionary lineage leading to humans from the various lineages leading to other mammalian species. The concentrations of the inhibitors, phenylalanine, homoarginine, phenylalanylglycylglycine, and levamisole, required to give 50% inhibition, [I_{50}], of the liver/bone/kidney/placental (nonhuman) alkaline phosphatases showed no significant variation among the species. However, the [I_{50}] values for the intestinal enzyme varied among species to a much greater extent. This implies that in the liver/bone/kidney/placental (nonhuman) alkaline phosphatase the structures of the binding sites for these inhibitors have been highly conserved during mammalian evolution, but there has been much greater divergence of these structures in the evolution of intestinal alkaline phosphatases.**

At least three gene loci determine the various forms of alkaline phosphatase [ALPase; orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] that occur in human tissues: one coding for the placental form, at least one coding for the intestinal forms (adult and fetal), and at least one coding for the liver, bone, and kidney forms (1–4). These three classes of ALPase are sharply discriminated from one another by quantitative inhibition with L-phenylalanine (Phe), L-homoarginine (Har), and L-phenylalanylglycylglycine (PheGlyGly) as inhibitors and also by their thermostabilities (1, 5, 6). In a recent study using these criteria we compared the ALPases occurring in liver and in placenta from a series of mammals with those occurring in human liver and placenta (7). We found that in each of the animal species studied the liver and placental ALPases closely resembled each other and were also very similar to human liver ALPase, but they differed from human placental ALPase. We have now extended these studies to include bone, kidney, and intestinal ALPases from the various species. We have also extended the battery of inhibitors to include levamisole, which is a particularly potent inhibitor of liver, bone, and kidney ALPase (8, 9).

We find that the liver, bone, kidney, and placental ALPases from the various species resemble each other and also human liver, bone, and kidney ALPases very closely, but they are sharply differentiated from the series of intestinal ALPases and from human placental ALPase. The results suggest that in these mammalian species there are two distinct forms of ALPase, a liver/bone/kidney/placental form and an intestinal form, which are presumably coded by separate gene loci. In humans a third distinct form occurs, human placental ALPase, coded by an additional gene locus. An unexpected finding is that although the species tested do not vary appreciably with respect to the concentrations of the inhibitors required to produce 50% inhibition, [I_{50}], of liver/bone/kidney/placental ALPases, the intestinal ALPases from the different species vary quite significantly in this respect. This implies that in the liver/bone/kidney/placental ALPases, the structures of the binding sites for these inhibitors have been highly conserved during mammalian evolution, but there has been much more divergence of these structures in the evolution of the intestinal ALPases.

MATERIALS AND METHOD

Liver, kidney, bone, placental, and intestinal samples were obtained from one or more representatives of the following species: pigs, cats, dogs, hamsters, cows, sheep, guinea pigs, mice, and rats. They were stored at −20°C until extracted. Aqueous solutions were obtained by butanol extraction as described (1). The intestinal extracts were heated at 56°C for 60 min to destroy any liver/bone/kidney ALPase that might be present (1). Inhibition studies were carried out as described (1, 7). The substrate was 5.0 mM *p*-nitrophenylphosphate (pH 9.8) and the inhibitors were Phe, Har, PheGlyGly, and levamisole. Inhibitions were performed with appropriate concentration ranges for the different inhibitors and the [I_{50}] for each ALPase and for each inhibitor was determined as described (7). Thermostability studies were carried out at 56°C and in some cases at 65°C as described (7). In each case the time required to produce 50% inactivation of the enzyme (T_{50}^{56} or T_{50}^{65}) was determined as described (7).

[I_{50}] and T_{50} determinations were routinely done in triplicate, and in most cases the determinations were repeated on other occasions with tissues from the same or other members of the particular species. The individual values for particular tissues in any one species given in Fig. 1 and in the various tables are averages of the whole set of determinations. Statistical analyses of these values were carried out with log [I_{50}] or log T_{50} values because it was found that log transformation tended to normalize the distributions being compared.

RESULTS

Fig. 1 shows the distributions of log [I_{50}] values for liver, bone, kidney, and placenta of the nine species with the four inhibitors. These ALPases are very sensitive to inhibition with levamisole ([I_{50}] $\simeq$ 0.03 mM), sensitive to Har ([I_{50}] $\simeq$ 2.7 mM), but relatively insensitive to Phe ([I_{50}] $\simeq$ 29 mM) and PheGlyGly ([I_{50}] $\simeq$ 31 mM). To examine the variation of [I_{50}] values for each inhibitor among the different tissues and among the different species, we carried out two-way analyses of variance. No sig-

Abbreviations: ALPase, alkaline phosphatase; [I_{50}], concentration of inhibitor required to produce 50% inhibition: T_{50}, time required to give 50% inactivation of ALPase; Har, homoarginine.

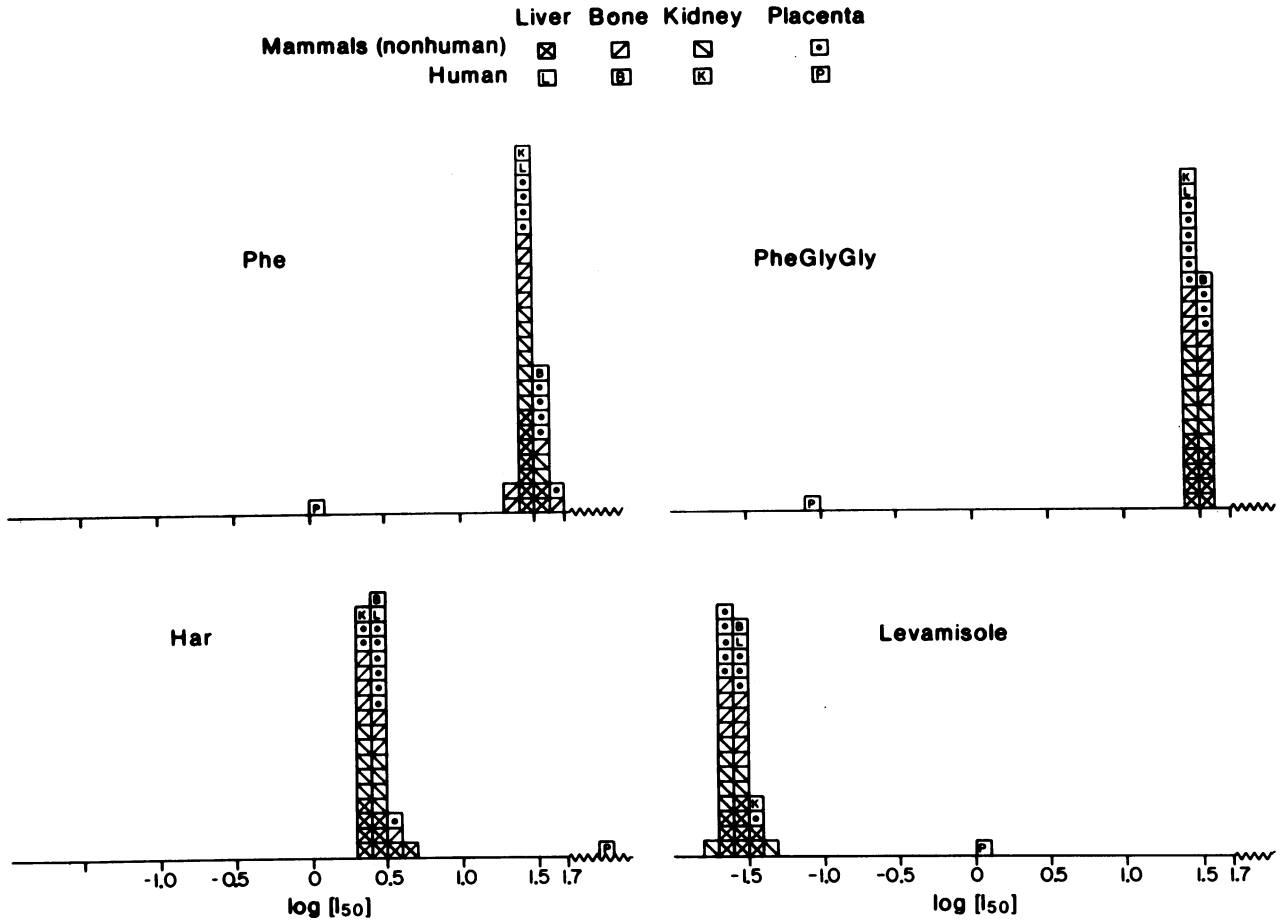

FIG. 1. Distributions of log [I_{50}] values for liver, bone, kidney, intestinal, and placental ALPases from nine mammalian species (pigs, cats, dogs, hamsters, cows, sheep, guinea pigs, mice, and rats) and also from humans.

nificant differences either among tissues or among species were detected. Fig. 1 also shows the corresponding human ALPases. The human liver, bone, and kidney [I_{50}] values fall within the range of values for the nine mammalian species, but the human placental ALPase is sharply differentiated.

Table 1 gives the T_{50}^{56} values for these various mammalian nonhuman ALPases. More variation occurs than with the [I_{50}] values, and a two-way analysis of variance indicates that there are significant differences among species ($F = 3.78$; df = 8, 24; $0.005 < P < 0.01$).

Table 2 gives the [I_{50}], T_{50}^{56}, and T_{50}^{65} values obtained for the various intestinal ALPases. In general, the inhibition profiles are quite different from these obtained for the liver, bone, kidney, and placental ALPases from these species. In particular, the intestinal ALPases are very much less sensitive to inhibition by Har or levamisole and, in eight out of nine cases, more sensitive to inhibition with Phe. No consistent difference is observed, however, with PheGlyGly because in some species the intestinal ALPase appears to be significantly more sensitive and in others less sensitive to inhibition with this inhibitor than are the corresponding liver, bone, kidney, and placental ALPases. It will also be seen (see Table 1) that the intestinal ALPases are usually much more thermostable than the corresponding liver, bone, kidney, and placental ALPases.

The [I_{50}] values for intestinal ALPase show a greater range of variation among species than is seen with the liver, bone, kidney, or placental ALPases. For Phe and levamisole this point can be examined by a direct comparison of the variances be-

Table 1. Time (min) at 56°C required to reduce ALPase activity to 50% of original activity (T_{50}^{56})

Species	Liver ALPase	Kidney ALPase	Bone ALPase	Placenta ALPase	Mean
Pig	4.4	12.4	7.4	9.7	8.5
Hamster	13.1	9.1	7.7	4.4	8.6
Cat	18.5	7.9	6.4	12.3	11.3
Dog	17.0	7.4	8.9	23.1	14.1
Cow	16.1	10.5	11.1	10.9	12.2
Sheep	15.4	12.2	8.8	10.8	11.8
Guinea pig	15.0	14.4	12.6	6.6	12.2
Rat	6.5	6.3	5.2	2.2	5.1
Mouse	7.5	6.7	4.2	2.3	5.2
Mean	12.6	9.7	7.6	9.1	

Table 2. [I_{50}] for Phe, Har, PheGlyGly, and levamisole and T_{50}^{56} and T_{50}^{65} for intestinal ALPase from nine mammalian species

Species	[I_{50}], mM				T_{50}^{56}, min	T_{50}^{65}, min
	Phe	Har	PheGlyGly	Levamisole		
Pig	2.1	>50	9.7	5.1	>60	7.0
Hamster	35.2	>50	>50	21.9	>60	13.6
Cat	3.7	>50	16.1	9.3	>60	66.3
Dog	3.5	>50	14.4	6.2	>60	16.9
Cow	14.0	>50	>50	13.5	>60	46.0
Sheep	10.2	>50	32.5	10.2	>60	15.9
Guinea pig	17.4	>50	>50	14.6	>60	6.7
Rat	12.3	>50	>50	23.7	35.3	2.0
Mouse	14.7	>50	>50	11.8	25.6	1.0

cause all the [I_{50}] values for intestinal ALPase are within the range in which satisfactory estimates of [I_{50}] can be made (i.e., <50 mM). These comparisons are shown in Table 3, which also shows a similar comparison of the variances of T_{50}^{65} values for intestinal ALPase and the T_{50}^{56} values for the liver/bone/kidney/placental nonhuman ALPases. The variance ratios (F) are in each case highly significant.

Table 4 summarizes the data and also gives data on the corresponding human tissues so that a number of critical comparisons can be made. The liver/bone/kidney ALPases of the nine mammalian (nonhuman) species do not differ significantly from the placental ALPases of these species or from human liver/bone/kidney ALPases in [I_{50}] values for the four inhibitors. There are, however, marked differences from the values for the intestinal ALPases. Human placental ALPase is sharply discriminated from the liver/bone/kidney/placental ALPases of the other species and from human liver/bone/kidney ALPase both by the [I_{50}] values for each of the inhibitors and also by the T_{50} values. It is sharply discriminated from the intestinal ALPases of the various species, including humans, by the [I_{50}] value with PheGlyGly and by the T_{50}^{65} value.

DISCUSSION

The findings suggest that two distinct forms of ALPase occur in most mammalian species, a liver/bone/kidney/placental form and an intestinal form. In humans, placental ALPase occurs as a third distinct form. Various thermostability and inhibition studies on some of the species we have examined here have been reported in the literature (8, 10–15). Despite differences in detailed methodology, the results obtained appear to be fully consistent with the present findings.

Relative sensitivities to inhibition with the four inhibitors used in the present study provide sharp discrimination among the three classes of enzyme. The differences in [I_{50}] values, on which this discrimination is based, presumably reflect structural differences in the binding site or sites for these substances on the enzyme molecules. The inhibitors act uncompetitively (9, 16, 17), so that the inhibitor-binding sites are presumably not identical with the substrate-binding site. No significant differences in [I_{50}] values for the four inhibitors were detected among the liver/bone/kidney/placental ALPases from the nine nonhuman species nor did they differ significantly from the corresponding values for the human liver/bone/kidney ALPases. This implies a considerable degree of conservation of the structure of inhibitor-binding site or sites of these enzyme molecules during mammalian evolution. For intestinal ALPase there is much greater variation of the [I_{50}] values among species than is found with liver/bone/kidney/placental (excluding human) ALPases. This suggests a greater degree of divergence of the structure of inhibitor-binding sites during the molecular evolution of intestinal ALPase in mammals.

Thermostability presumably reflects overall molecular structure in some way and so it is perhaps not surprising that the T_{50} values should reveal significant differences among species both for the liver/bone/kidney/placental (nonhuman) ALPases and also the intestinal ALPases. However, the intestinal ALPases show a greater degree of variability among the species than the liver/bone/kidney/placental ALPases.

We have previously suggested that the three gene loci presumed to be coding for the liver/bone/kidney, intestinal, and placental ALPases in humans were in the course of evolution derived by successive gene duplications from a common ancestral gene (7, 15). Subsequent to each duplication, divergence by the fixation of point mutations was presumed to have occurred, thus accounting for the differences in specific characteristics, such as relative sensitivity to particular inhibitors, that we now observe among the enzyme products of the different loci. The remarkable differences between human placental ALPase and the ALPases present in placentas of various animal species led to the further hypothesis that the duplication from which the gene locus coding for human placental ALPase originated was a late evolutionary event that occurred subsequent to the divergence of the evolutionary lineage leading to humans from the various lineages leading to other mammalian

Table 3. Variances (V) of log [I_{50}] values for Phe and levamisole and of log T_{50} values for intestinal and liver/kidney/bone/placental ALPases in nine nonhuman mammals

		log [I_{50}]			
		Phe	Levamisole	log T_{50}^{56}	log T_{60}^{65}
Itestinal ALPase*	V_1	0.1568	0.0650	—	0.3457
Liver/kidney/bone/placental ALPase†	V_2	0.0036	0.0050	0.0538	—
Variance ratio $F = V_1/V_2$		43.55	11.82	6.43	
Significance (P)		<0.001	<0.001	<0.001	

* Nine species; df = 8.

† Nine species; four tissues; df = 35.

Table 4. Comparisons of means ($\bar{x}$), standard deviations (SD x), means of logs ($\overline{\log x}$), and standard deviations of logs (SD log x) of [I_{50}] for four different inhibitors and T_{50}^{56} and T_{50}^{65} of liver/kidney/bone ALPases, placental ALPase, and intestinal ALPase from nine mammalian species and from humans*

ALPase		[I_{50}], mM				T_{50}^{56}, min	T_{50}^{65}, min
		Phe	Har	PheGlyGly	Levamisole		
Liver/kidney/bone							
Nine mammals	$\bar{x}$	29.9	2.6	31.4	0.028	10.1	—
(n = 27)	SD x	4.3	0.44	3.9	0.005	4.1	
	$\overline{\log x}$	1.47	0.41	1.49	−1.57	0.97	—
	SD log x	0.06	0.06	0.05	0.08	0.18	
Human	$\bar{x}$	31.0	2.7	30.6	0.033	7.4	—
(n = 3)	SD x	6.3	0.15	5.2	0.006	1.5	
	$\overline{\log x}$	1.49	0.43	1.48	1.48	0.86	—
	SD log x	0.09	0.03	0.07	0.07	0.09	
Placental							
Nine mammals	$\bar{x}$	33.1	2.7	30.8	0.026	9.1	—
(n = 9)	SD x	4.2	0.27	3.3	0.003	6.5	
	$\overline{\log x}$	1.52	0.44	1.49	−1.59	0.85	
	SD log x	0.05	0.04	0.05	0.06	0.34	
Human	x	1.1	>50	0.08	1.2	—	≫60
(n = 1)	log x	0.41	>1.7	−1.10	0.08		≫1.78
Intestinal							
Nine mammals	$\bar{x}$	12.6	>50	—	11.8	—	19.5
(n = 9)	SD x (or range)	10.1		(14.4–>50)	6.9	(25.6–>60)	22.1
	$\overline{\log x}$	0.96	>1.7	—	1.01	—	1.00
	SD log x	0.40	—	1.16–>1.7	0.26	(1.41–>1.77)	0.59
Human	x	1.0	>50	5.3	3.4	>60	6.5
(n = 1)	log x	0.00	>1.7	0.72	0.53	>1.78	0.81

* Note that for the four inhibitors and the liver/bone/kidney/placental ALPases of the nine mammalian species, SD x is highly correlated with $\bar{x}$, but SD log x is essentially independent of $\overline{\log x}$.

species. An alternative hypothesis is that the gene locus for human placental ALPase does exist in these other mammalian species, but is not expressed in their placentas or indeed in other tissues so far examined so that the late evolutionary event would have involved its derepression or activation leading to its expression in placenta (7).

Whatever the nature of the evolutionary change that gave rise to the appearance of human placental ALPase, it will be of obvious interest to attempt to date the time when it first appeared by investigating the ALPases in placenta and other tissues of various primates. It has already been reported that in chimpanzees and orangutans, the placental ALPase is similar to human-type placental ALPase (18). We have confirmed this result by thermostability, inhibition, and immunochemical studies. Our preliminary results suggest, however, that in gibbons as well as in various other less closely related primates the ALPase expressed in placenta is of the liver/bone/kidney type. If this is confirmed by further work, it suggests that the critical evolutionary event resulting in the appearance of human-type placental ALPase occurred subsequent to the divergence of the evolutionary lineage leading to the Hylobatidae (lesser apes), but prior to the divergence of the lineage leading to the Pongidae (greater apes).

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