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Genetics

Lack of homology between dog and human placental alkaline phosphatases

(inhibition/thermostability/electrophoresis/evolution/duplication)

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ABSTRACT **Alkaline phosphatases [ALPases; orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] from dog and human placenta, liver, bone, kidney, and intestine were investigated by inhibition studies with L-homoarginine, L-phenylalanine, and L-phenylalanylglycylglycine; by thermostability studies; and by electrophoresis, both before and after treatment with neuraminidase. The inhibitions obtained for each inhibitor with dog placental ALPase closely match those obtained with dog and human liver, bone, and kidney ALPases, but are quite different from those obtained with human placental ALPase. Dog placental ALPase is thermolabile, as are dog and human liver, bone, and kidney ALPases, in marked contrast to human placental ALPase, which is very thermostable. Dog placental ALPase has the same electrophoretic mobility as dog liver, bone, and kidney ALPases after removal of sialic acid residues with neuraminidase. Desialated human placental ALPase differs electrophoretically from desialated human liver, bone, and kidney ALPases, which show the same mobilities. Dog and human intestinal ALPases are distinguished by these various criteria from the liver, bone, kidney, and placental ALPases of both species, but are similar to each other. These results suggest that the ALPase gene locus expressed in dog placenta is not homologous to that expressed in human placenta. Rather, it appears to be homologous to the ALPase locus expressed in dog and human liver and possibly also bone and kidney. Other incomplete data suggest that this may also be true for placental ALPase in other mammalian species. One possible explanation is that human placental ALPase, a relatively recent newcomer on the evolutionary scene, arose from a gene duplication that occurred subsequent to the evolutionary divergence of many other mammalian species.**

The alkaline phosphatases [ALPases; orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] are a group of similar enzymes that hydrolyze various monophosphate esters. They are glycoproteins (1–4) named for the different tissues in which they predominate, the best characterized forms being those from liver, bone, kidney, intestine, and placenta (5). Several lines of evidence (summarized in refs. 6 and 7) suggest that in man the various ALPases are determined by at least three gene loci: one coding for the placental enzyme, at least one coding for the intestinal enzyme, and at least one coding for the liver, bone, and kidney forms of the enzyme. In the case of intestinal ALPase, electrophoretic differences between adult and fetal forms occur (7), and, in the case of liver and bone ALPases, thermostability (8, 9) and immunologic differences (10, 11) have been reported. It is not yet clear in either of these cases whether posttranslational changes or separate loci account for these differences.

Various techniques have been used to distinguish between the three main types of human ALPase. They can, for example, be differentiated by inhibition studies with certain amino acids and peptides (6), by thermostability studies (8, 9, 12), and by electrophoresis after treatment of the ALPase with neuraminidase, which removes sialic acid residues (13). We have applied these techniques to ALPases from different tissues of the dog and find that dog placental ALPase closely resembles dog liver, bone, and kidney ALPases and also human liver, kidney, and bone ALPases in its characteristics; but it is sharply distinct from human placental ALPase and also from dog and human intestinal ALPases. The data suggest that the ALPase gene locus expressed in dog placenta is not homologous with that expressed in human placenta, but is probably homologous with the locus expressed in human liver and possibly also bone and kidney. The findings raise new and interesting questions about the molecular evolution of ALPases.

MATERIALS AND METHODS

Dog liver, kidney, bone, and intestinal samples were obtained from four beagles and one mixed breed. Fourteen dog placentas were obtained from two litters of beagles. Human liver, kidney, bone, and intestinal samples were obtained from several autopsies conducted within 12 hr of death. Meconium obtained from diapers of newborns was used as a source of human fetal intestinal ALPase. Human placentas were obtained from normal deliveries. The tissue samples were stored at −20°C if not extracted immediately. Tissue extracts were made by a modification of the butanol method of Morton as described (6). ALPase activity was determined spectrophotometrically at 30°C with 5.0 mM *p*-nitrophenyl phosphate/1.0 M diethanolamine (pH 9.8) as described (6). Inhibition studies were carried out by the same procedure except for the inclusion of 0.5, 1.0, 5.0, 10.0, or 20.0 mM inhibitor in the reaction mixtures. The inhibitors used were L-homoarginine (Calbiochem), L-phenylalanine, and L-phenylalanylglycylglycine (Sigma). Degree of inhibition was expressed as a percentage of the original activity remaining in the presence of the particular concentration of the inhibitor. Thermostability studies were carried out on extracts previously dialyzed against 0.01 M Tris·HCl, pH 7.5/1.0 mM $MgCl_2$. The extracts were diluted with this buffer to give a convenient activity for the assay, and 1 mg of bovine serum albumin per ml was added. Aliquots (0.2 ml) of the extracts were heated in a waterbath in covered glass tubes at 65°C for varying periods up to 50 min and then placed in an icebath where control samples had been kept for the same amounts of time. Starch gel electrophoresis of the tissue extracts was carried out at 5°C using a Tris borate discontinuous buffer system, pH 8.0–8.6, as described (13). Neuraminidase treatment of the ALPases was performed by mixing a volume of sample (0.5 ml) with an equal volume of 10.0 mM Na_2HPO_4/citrate buffer, pH 5.0, containing 1 unit of neuraminidase (Sigma type VI) and incubating the mixture for 60 min at 37°C.

Abbreviation: ALPase, alkaline phosphatase.

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RESULTS

Inhibition Studies. Fig. 1 summarizes the results obtained in inhibition studies with L-homoarginine, L-phenylalanine, and L-phenylalanylglycylglycine as inhibitors. These inhibitors were chosen because previous work had shown that together they provide a particularly clear discrimination between human placental, intestinal, and liver/bone/kidney ALPases (6). The main findings are as follows: (*i*) L-Homoarginine produced marked inhibition of dog placental ALPase, and the inhibition curves were similar to those for liver/bone/kidney ALPases of both dog and man. In contrast, human placental, dog intestinal, and human intestinal ALPases were much less inhibited. (*ii*) L-Phenylalanine produced marked inhibition of human placental and intestinal ALPase. Dog intestinal ALPase was also strongly inhibited but to a lesser degree. However, dog placental ALPase and dog and human liver/bone/kidney ALPases exhibited only a modest degree of inhibition, and their inhibition curves matched each other closely. (*iii*) L-Phenylalanylglycylglycine produced very marked inhibition of human placental ALPase and only a modest degree of inhibition of dog placental ALPase. The inhibition curve for dog placental ALPase closely matched those for both dog and human liver/bone/kidney ALPases. Human and dog intestinal ALPases were inhibited to a lesser degree than human placental ALPase but to a somewhat greater degree than dog placental ALPase and dog and human liver/bone/kidney ALPases.

Thus, in its behavior with these different inhibitors, dog placental ALPase closely resembles dog liver, bone, and kidney ALPases and also human liver, bone, and kidney ALPases, and the pattern of inhibition is clearly distinct from that for human placental ALPase. Dog and human intestinal ALPases differ in inhibition pattern both from the placental and the liver/bone/kidney ALPases in each of the species. Van Belle (14) has shown that dog placental ALPase behaves in the same way as dog liver, bone, and kidney ALPases with L-phenylalanine and also with another ALPase inhibitor, levamisole.

Thermostability Studies. Human placental ALPase is remarkably thermostable. It may be heated at 65°C for periods of up to 1 hr with little or no loss of activity. In contrast, human liver, bone, kidney, and intestinal ALPase activities are rapidly destroyed under these conditions. Human intestinal ALPase is less thermolabile at 56°C than human liver, bone, or kidney ALPases (12). Also, bone ALPase is slightly more heat-labile than liver ALPase (9). We have found that the same general pattern of differential thermolabilities occurs with dog liver, bone, and intestinal ALPases. However, dog placental ALPase closely resembles dog liver ALPase in this respect. The striking difference in thermolabilities between dog and human placental ALPases is illustrated in Fig. 2.

Electrophoretic Studies. Electrophoretic studies have shown that the ALPase isozymes from extracts of human liver, bone, and kidney after they have been treated with neuraminidase have essentially the same mobilities, although the mobilities of the untreated ALPase isozymes differ one from another (13). Thus, most of the electrophoretic differences between the untreated liver, bone, and kidney ALPases can be attributed to differences in their sialic acid contents. We have observed essentially the same phenomenon with dog liver, bone, and kidney ALPases. The electrophoretic mobilities of human placental ALPase isozymes are also retarded after treatment with neuraminidase, although to a much lesser degree than the liver, bone, and kidney ALPases, so that the two sets of ALPase isozymes are clearly separated (13). Fig. 3 shows that the electrophoretic mobility of dog placental ALPase was unaffected by treatment with neuraminidase, presumably because it lacks sialic acid residues. However, it had the same mobility as dog liver, bone, and kidney ALPases after they had been treated with neuraminidase.

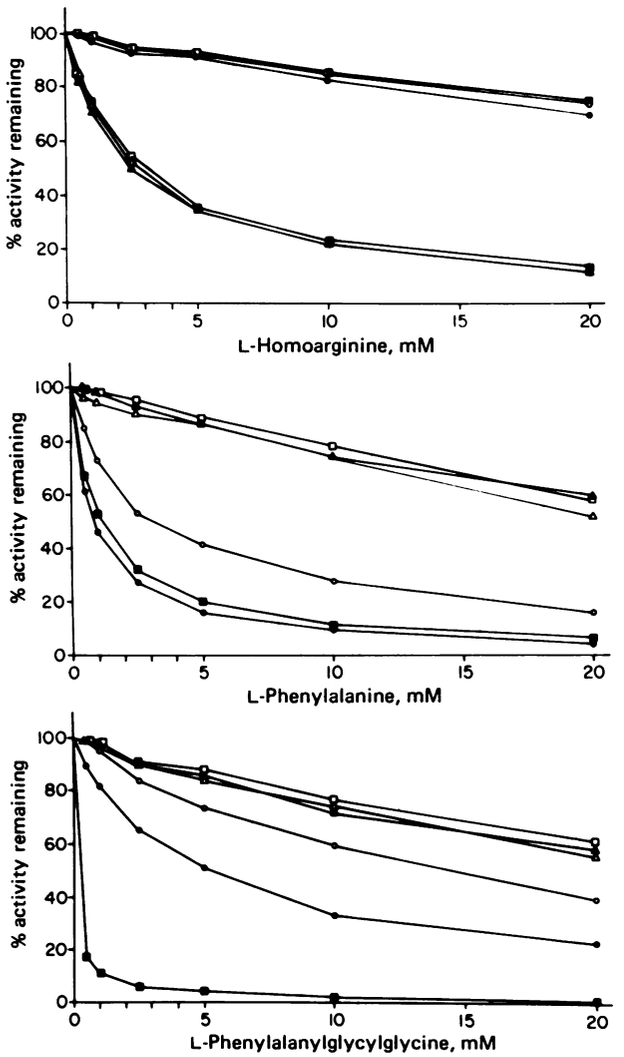

FIG. 1. Inhibition of dog and human placental, intestinal, liver, bone, and kidney ALPases by L-homoarginine, L-phenylalanine, and L-phenylalanylglycylglycine. □, Dog placenta; ■, human placenta; ○, dog intestine; ●, human intestine; △, dog liver, bone, and kidney; ▲, human liver, bone, and kidney. The data points for the liver, bone, and kidney ALPases were combined for each species because of their similarity.

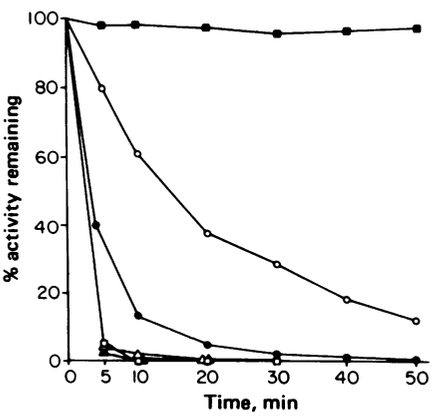

FIG. 2. Effect on ALPase activity of heating dog and human placental liver and intestinal ALPases at 65°C for different times. Symbols as in Fig. 1.

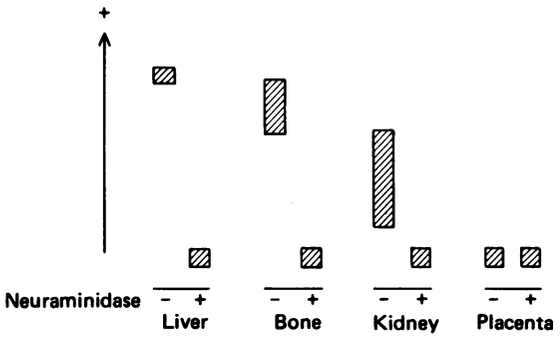

FIG. 3. Relative electrophoretic mobilities of dog liver, bone, kidney, and placental ALPases before (−) and after (+) treatment with neuraminidase.

Adult and fetal human intestinal ALPases differ in electrophoretic mobility, although they have an identical pattern of inhibition with various inhibitors and similar thermostabilities (7). The electrophoretic mobility of human adult intestinal ALPase is unaffected by neuraminidase treatment. Human fetal intestinal ALPase has a greater anodal mobility than the adult intestinal enzyme and is retarded somewhat by neuraminidase treatment. But, even after this treatment, its mobility is slightly greater than that of the adult form. Five different samples of dog intestinal extracts were examined. In each, significant retardation of the ALPase after neuraminidase treatment was observed, but the mobility even after exhaustive neuraminidase treatment was not the same in all samples, two having faster mobilities than the other three. Whether this represents a genetic polymorphism, analogous to that known to occur with human placental ALPase but not found with human liver, bone, kidney, or intestinal ALPases, is at present uncertain.

DISCUSSION

Perhaps the most striking results are those provided by the inhibition studies. It has previously been shown that these particular inhibitors discriminate sharply among human placental, intestinal, and liver/bone/kidney ALPases. However, dog placental ALPase shows an inhibition pattern virtually identical to that of dog and human liver/bone/kidney ALPases. This inhibition pattern is quite different from that obtained with human placental ALPase. ALPase inhibition by L-phenylalanine and L-homoarginine is uncompetitive (15, 16), and therefore presumably involves binding sites for these inhibitors that are not identical with the substrate binding site of the enzymes. The binding site or sites for L-phenylalanine, L-homoarginine, and L-phenylalanylglycylglycine in dog placental ALPase must be similar to those in both dog and human liver, bone, and kidney ALPases, but different from the site or sites for these inhibitors in human placental ALPase. This implies a significant difference in enzyme structure between dog and human placental ALPases and a similarity in the structures of dog placental and both dog and human liver, bone, and kidney ALPases.

The thermostability and electrophoretic findings support this conclusion. Human placental ALPase is remarkably thermostable, whereas dog placental ALPase is by comparison very thermolabile and, in this respect, resembles dog and human liver, bone, and kidney ALPases. Dog placental ALPase has essentially the same electrophoretic mobility as desialated dog liver, bone, and kidney ALPases, whereas desialated human placental ALPase is different electrophoretically from desialated human liver, bone, and kidney ALPases. Thus, the results as a whole indicate that dog placental ALPase is more closely related to human liver, bone, and kidney ALPases in its structure and properties than it is to human placental ALPase. In both species, the intestinal enzymes differ in their properties from the placental and liver, bone, and kidney enzymes, but in a number of respects are similar to each other.

It has been pointed out that very often two or more separate gene loci code for a set of enzyme proteins which, though structurally distinct, are very similar in their catalytic properties and also in their subunit structures (17). In evolutionary terms, the most plausible explanation for this common phenomenon is that in each case the two or more genes involved are descended from a common ancestral gene which at some point in the course of evolution was duplicated. A later duplication of one or other of the first pair would account for the occurrence of three loci and so on. That the enzyme products of the different loci, although very similar, now differ in some degree in their primary structures and their enzymic and physical properties can be readily accounted for in terms of subsequent mutations in the original duplicate genes and their consequent divergence.

In many such multilocus enzyme systems, expression of the several loci may differ markedly from tissue to tissue or in different compartments of the same cells within a tissue. When this occurs, the enzyme product of a locus expressed in a particular tissue or cell compartment in one mammalian species generally appears to resemble more closely the enzyme formed in the corresponding tissue or cell compartment in another mammalian species than it resembles the enzyme products of the other loci in the particular multilocus set. This suggests that, as a general rule, homologous loci in such a multilocus set are expressed similarly in the same tissues of different species. It is in this connection that the data presented here are of special evolutionary interest. The liver, bone, and kidney ALPases of dog and man resemble each other so closely in their general characteristics that it is reasonable to infer that they represent the expression of homologous gene loci in the different tissues of the two species. Similarly, the intestinal enzymes in the two species probably represent the expression of homologous loci. Because of mutations at these loci in the course of evolution, one would not expect the structure and properties of these enzymes in the two species to be exactly the same even though the same gene locus is involved. But the marked difference between the placental ALPases in man and dog and the similarity of the dog placental ALPase to the dog and human liver ALPases appears to constitute a clear exception.

In effect, the data suggest that the ALPase gene locus expressed in dog placenta is not homologous to that expressed in human placenta. Rather it appears to be homologous to the locus expressed in dog and human liver, and possibly also in bone and kidney. Other data suggest that this may also be true for the placental enzyme in a number of other mammalian species. For example, we have found that cow and pig placental ALPases resemble cow liver, dog and human liver, bone, and kidney, and dog placental ALPases in their inhibition patterns with L-homoarginine, L-phenylalanylglycylglycine, and L-phenylalanine. It has been reported that the ALPase in placentas from rat, rabbit, hamster, guinea pig, mouse, rhesus monkey, baboon, and African green monkey are much less sensitive to L-phenylalanine inhibition compared with the human placental enzyme (18, 19). Furthermore, in the rat, liver and placental ALPases cannot be distinguished by L-phenylalanine or levamisole inhibition (20). Similarly, the placental enzymes from these and a number of other mammalian species have been found to be much more thermolabile than human placental ALPase (18, 19, 21).

Much more detailed information about placental ALPase in different mammalian species is needed before any firm conclusion can be reached. But the apparent lack of homology between human placental ALPase and other mammalian placental ALPases raises questions of unusual evolutionary interest. One possibility that should be considered is that human placental ALPase is a relatively recent newcomer on the evolutionary scene and arose from a gene duplication that occurred subsequent to the divergence of many other mammalian species. Thus, we may be witnessing the evolution of a particular enzyme protein by mutation at a relatively new structural locus. It is of interest to note in this connection that in man placental ALPase is highly polymorphic, and, indeed, a much greater incidence of allelic variants has been found for this enzyme than in the case of any other enzyme so far studied (22). In contrast, polymorphism has not been detected in the other human ALPases.

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