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Review

Bacterial Amidinohydrolases and Their Plausible Evolutionary Relationships

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Molecular and enzymic properties of bacterial amidinohydrolases were compared. The enzymes which hydrolyze ω-guanidino acids were classified into two types, and further studies with respect to inhibitor specificity and electrophoretic behavior strongly suggested that the enzymes of the same type bare evolutionary relationships.

KEY WORDS: Amidinohydrolases / Comparison of enzymes / Evolution of enzyme / Guanidino acids /

INTRODUCTION

Comparative studies on the primary structure of specific proteins from various sources have provided the raw material for speculations on the nature of the variations which have occurred at the biochemical level during evolution. The variable features of specific proteins, cytochrome C for example,[1,2] have been evidenced. They must represent a partial record of the interplay of genetic variability and natural selection on the structural gene of the protein, during the evolutional divergence of the organisms containing these proteins. A large number of isozymes has also been investigated in a wide variety of organisms and there has been an increasing awareness that enzyme multiplicity is the rule rather than exception in eucaryotes.[3] There is plenty of evidence which indicates that functionally similar isozymes were developed from a common ancestral protein in the process of evolution. Amino acid sequence studies have revealed the structural homology between lysozyme and α-lactoalbumin,[4] among serine proteases,[5] and several other enzymes of different functions.[6] To account for the evolutional differentiation of an enzyme to give rise to another enzyme with new activity, the involvement of the following two steps have been proposed; (1) duplication of a gene for an ancestral enzyme, and (2) mutational change of substrate specificity and other properties.

There is a wide variety of catabolic pathways in bacteria which are involved in the degradation of many biological compounds. For example, a wide variety of degradation pathway of L-lysine[7] or L-arginine[8] has been well documented. This is in contrast to the relative unity of their biosynthetic routes. This fact indicates that the degradative pathways have evolved in each bacterium separately and independently, followed by selection imposed by the nutritional opportunities. The pseudomonads and other soil bacteria are active participants in the process of mineralization of organic matter in nature with catabolic versatility. We can assume

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that there is a microbial degradative sequence for every biochemical compound in existence.

There is a growing interest in the evolution of metabolic pathways and enzymes in bacteria. Many attempts in experimental evolution represent a likely approach to the solution of pollution problems resulting from the enterprise of chemical industries.[9,10] Another approach to the problem is the comparison of natural enzymes which catalyze analogous reactions. A number of reviews have been published dealing with evolution of microbial enzymes from a variety of stand points.[11-17] Jensen has summarized recent studies on enzyme recruitment of new function in evolution, emphasizing the prospect for defining evolutionary relationships between proteins that coexist in the same organism but that no longer serve the same function.[15]

Amidinohydrolases hydrolyze guanidino compounds to form the corresponding amino compounds and urea. The most well-known amidinohydrolase is mammalian arginase. In bacteria several amidinohydrolases have been found. Extensively studied amidinohydrolases are the enzymes specifically hydrolyse creatine,[18] L-arginine,[19-22] guanidinosuccinate,[23] guanidinoacetate,[24,25] 3-guanidinopropionate[26,27] and 4-guanidinobutyrate.[26-32] Amgatine amidinohydrolase[33] and methyl guanidine amidinohydrolase[34] are also known. Among substrates of the bacterial amidinohydrolases, shown in Fig. 1, guanidinoacetate, 3-guanidinopropionate, and 4-guanidinobutyrate provide a homologous series of ω-guanidino monocarboxylic acids and three bacterial strains have been selected in our laboratory, each of which can produce two of the three enzymes acting on the series of compounds; guanidinoacetate amidinohydrolase (GAH, EC 3.5.3.2), 3-guanidinopropionate amidinohydrolase (GPH, EC 3.5.3.-) and 4-guanidinobutyrate amidinohydrolase (GBH, EC 3.5.3.7). The six amidinohydrolases from the three bacterial strains are all inducible and their crude extracts exhibit specific activities moderate for enzyme purification.

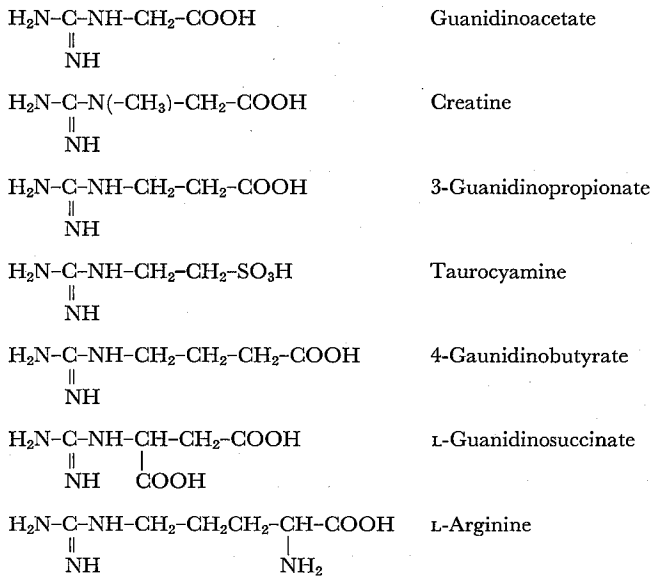

Fig. 1. Substrates of bacterial amidinohydrolases.

This situation fulfils the fundamental prerequisites for comparative studies on analogous enzymes; high or clear-cut substrate specificity of enzymes, analogy of reactions catalyzed, and facility in prepararing purified enzymes. Some of these enzymes have been purified extensively in our laboratory.

This article will propose a hypothesis that these enzymes can be classified into two types, type I and type II, on the basis of molecular weights, metal requirements and some other properties and suggests evolutionary relationships between enzymes of the same type from the results of direct comparisons of the properties of some sets of enzymes of different functions. The properties of some other bacterial amidinohydrolases will also be given, though primary emphasis be placed on the comparative aspects.

DISTRIBUTION

(1) Bacterial Strains, Each Produces Two Analogous Amidinohydrolases

Pseudomonas sp. ATCC 14676 produces GAH[24] and GBH.[31] *Pseudomonas aeruginosa* strain PAO1 (ATCC 15692), which produces GPH[26,27] and GBH,[26] was initially isolated by Holloway[35] and has been used in many laboratories for studies on genetic organization of pseudomonads. It was fortunate, therefore, to find the ability of the strain to produce the two analogous enzymes. We could expect that combination of studies on the gene loci of the two enzymes and those on the properties and structures of the enzymes will provide evidence as to some features in the evolution of bacterial enzymes such as significance of gene duplication or gene transfer. *Flavobacterium* GE-1 was isolated from garden soil as a potent producer of GAH and found later to be able to produce GBH. In early studies it was found that the properties of *Flavobacterium* GAH were significantly different from those of *Pseudomonas* GAH, and this fact led our studies to compare the other amidinohydrolases produced by these two organisms.

(2) Other Amidinohydrolase-Producing Bacteria

Pseudomonas putida P2 (ATCC 25571) was used for the studies on L-arginine catabolism in which GBH is involved. *Pseudomonas putida* var. *naraensis* C-83 was used for the studies on creatinine metabolism in which creatine amidinohydrolase is involved. *Pseudomonas chlororaphis* was isolated from garden soil for the studies on metabolism of guanidinosuccinic acid. Its guanidinosuccinate amidinohydrolase was purified and used for the determination of the configuration of guanidinosuccinic acid isolated from urine of patients with uremia. Arginase was found in *Bacillus subtilis*[19,21] and the sporulating cells of *B. licheniformis*.[20] Arginase of *B. subtilis*[21] has proved to be involved in a regulation mechanism comparable to that of yeast arginase.[37] The regulation of arginase associated with ornithine carbamoyltransferase has been reviewed by Wiame,[36] and this is beyond the scope of this article.

MOLECULAR WEIGHTS AND ENZYME TYPES

Table I shows the molecular weights and some other properties of GAH, GPH,

Table I. Some Properties of Purified Bacterial Amidinohydrolases

Substrate	Organism	Purity	Specific activity	Metal	Opt. pH	*Km* (mM)	M.W. $\times 10^{-4}$	Subunit $\times 10^{-4}$	Type	Ref.
Creatine	*Pseudomonas putida*	Cryst.	0.128	—	8.0	1.33	9.4	4.7		18
Guanidinoacetate	*Pseudomonas* sp. ATCC 14676	Cryst.	63.7	Mn^{+2}	9.0 ~9.5	9.1	16.3	3.8	I	24
Guanidinoacetate	*Flavobacterium* GE-1	Cryst.	190	Zn^{2+}, Co^{2+}	8.0 ~8.5	15.0	28.1 (24.2)[b]	7.0 (4.2)[b]	II	25
3-Guanidinopropionate	*Pseudomonas aeruginosa* PAO1	Cryst.	629	Mn^{2+}	9.0	45.5	19.5 ~21.5	3.4	I	26, 27
4-Guanidinobutyrate	*Pseudomonas* sp. ATCC 14676	Homog.	252	Mn^{2+}	9.5	33.0	18.0 ~18.6	3.3 ~3.6	I	29
4-Guanidinobutyrate	*Pseudomonas putida*	60 %	303[a]	Mn^{2+}	10.0	32.0	17.8 ~19.0	—	I	32
4-Guanidinobutyrate	*Flavobacterium* GE-1	Homog.	362	Zn^{2+}, Co^{2+}	8.0 ~8.5	2.16	24.2	4.2	II	30
Guanidinosuccinate	*Pseudomonas chlororaphis*	Homog.	9.73	Co^{2+}	8.0	8.3	30.0	—	II	23

a) The values are not corrected for the purity.

b) The values in the parentheses were obtained by re-examination.[28]

GBH, and other two amidinohydrolases. The table also shows a hypothetical classification of these enzymes into two types; type I and type II. As described below, the enzymes assigned to the same type have similar molecular and catalytic properties in spite of their different substrate specificities. The molecular weights of type I enzymes (Mn^{+2}-dependent enzymes) range from 163,000 to 215,000*. Type II enzymes (Zn^{2+} or Co^{2+}-dependent enzymes) have the molecular weights ranging from 242,000 to 300,000. Animal and microbial arginases seem to comprise two groups. One group consists of enzymes called light class arginases including "ureotelic" arginases; the molecular weights of mammalian liver arginases range from 110,000 to 150,000,[37-39,44,45] and the value for a yeast enzyme is 114,000.[37] The other group contains enzymes called heavy class arginases including "uricotelic" arginases;[40] the molecular weight of chicken liver arginase is 276,000,[40] and the values determined for the enzyme of *Neurospora classa*, *Bacillus subtilis*, and *B. licheniformis* are 278,000,[41] 276,000,[21] and 260,000,[22] respectively. The molecular weight of *B. subtilis* KY 3281 was determined to be 115,000,[19] being not studied further on the discrepancy.

As shown in Table I, the subunit molecular weight of *Pseudomonas* GAH, a type I enzyme, is 38,000. The other type I enzymes have subunit molecular weights in a range from 33,000 to 35,000. These values are comparable to those for arginases of either group. *Saccharomyces cerevisiae* arginase is trimer of subunit, the molecular weight of which is 39,000.[37] Rat liver arginase subunit has a molecular weight of 36,500.[39] The subunit molecular weight of *B. licheniformis* arginase, a heavy class arginase, is 33,000,[22] and the hexameric structure for the enzyme has been suggested. GAH and GBH of a *Flavobacterium*, type II amidinohydrolases, have somewhat larger subunit sizes of 42,000 molecular weight.

It should be noted that arginases and type I amidinohydrolases have similar subunit sizes and share Mn^{2+}-dependence in common. Additional studies on the structural relationships among these enzymes might yield important information about evolution of enzymes. More detailed comparisons of molecular weights and subunit sizes of bacterial type I and type II amidinohydrolases will be described later.

CATALYTIC PROPERTIES

(1) Metal Requirement

The metal requirement of creatine amidinohydrolase has not been reported. Arginase is well known to be a metalloenzyme requiring Mn^{2+} as an essential cofactor. This is true for enzymes of mammalian and microbial origin. As for bacterial amidinohydrolases other than arginase, two types of metal requirement have been revealed. Type I amidinohydrolases require Mn^{2+} and type II enzymes do Zn^{2+} or Co^{2+} for their catalytic activity.

GAH of *Pseudomonas* sp. ATCC 14676 is inhibited by EDTA, with which the

* The properties of GBH of *P. aeruginosa* PAO1 have not been published. The purification of this enzyme is in progress in our laboratory.

bound metals can be dissociated with relative ease.[24] GBH of the same strain[31] and GPH of *P. aeruginosa* PAO1[27] are not inhibited by EDTA or *o*-phenanthroline in Tris-HCl buffer at pH 8.0 or above. For the dissociation of these enzymes with EDTA is required incubation in phosphate buffer at pH below 7.0 and at an elevated temperature of 50°C or above. GAH[25] and GBH[30] of *Flavobacterium* GE-1, type II enzymes, also have tightly bound metals. Among metal chelators tested, only *o*-phenanthroline was effective, and incubation at 50°C for 30 min was required for a nearly complete inactivation of GAH. A significant irreversible inactivation was observed with GBH. Incubation of both enzymes with the chelator at 30°C resulted in no loss of the activity. The both *o*-phenanthroline-treated enzymes were activated with Zn^{2+} or Co^{2+}; Zn^{2+} was more effective. Gaunidinosuccinate amidinohydrolase, a type II enzyme, was reported to lose its activity by about 70 % during ammonium sulfate fractionation and the lost activity was restored by incubation with Co^{2+}. Zn^{2+} and other divalent metals were reported to be inactive when tested at a concentration of 10 mM.[23] Considered in conjugation with the effects of metal salts on the other type II enzymes, the metal concentration employed for guanidinosuccinate amidinohydrolase might be too high to exclude the possibility that Zn^{2+} also acts as an activator. The optimal concentrations of Zn^{2+} or Co^{2+} for the two *Flavobacterium* enzymes are in a range of 20–50 μM and a certain degree of inhibition is observed at higher concentrations.

(2) Binding Specificity

In general, the purified microbial amidinohydrolases show relatively high substrate and inhibitor specificities. L-Canavanine is a good substrate of bovine liver arginase, but is a poor substrate of heavy class arginase of chicken or *Neurospora classa*.[41] L-Ornithine and L-lysine are the competitive inhibitors of mammalian arginase but only L-lysine inhibits *Bacillus subtilis* arginase.[19] GAH of *Pseudomonas* sp. ATCC 14676 does not act on the higher homologs of guanidinoacetate, *i.e.*, 3-guanidinopropionate and 4-guanidinobutyrate.[24] GPH of *Pseudomonas aeruginosa* hydrolyzes 4-guanidinobutyrate and taurocyamine with relative reaction rates of 3.3 % and 11 %, respectively, but does not act on guanidinoacetate.[27] GBH of *P. putida* hydrolyzes 5-guanidinovalerate and 6-guanidinocaproate with relative rates of 11 % and 6 %, respectively, but does not act on the lower homologs of 4-guanidinobutyrate.[32] GBH of *Pseudomonas* sp. ATCC 14676 exhibits a similar specificity with a slightly lower rate for 5-guanidinovalerate of 5 %.[31] Thus, the specificities of type I enzymes other than GAH are relatively low for higher homologs of the normal substrates but very high for the lower homologs. The substrate specificity of *Flavobacterium* GAH, a type II enzyme, is somewhat lower than that of GBH of the same type, in contrast to the high specificity of GAH of type I. Type II GAH hydrolyze 3-guanidinopropionate and 4-guanidinobutyrate at relative rates of 19 % and 9 %, respectively,[25] while type II GBH exhibits essentially no activity toward higher and lower homologs of the substrate.[30] Guanidinosuccinate amidinohydrolase hydrolyzes N-amidino-L-glutamate, the next higher homolog of guanidinosuccinate, at a relative rate of about 4 %.[23]

Information about competitive inhibitors of amidinohydrolases is important as these compounds bind to the active sites but do not undergo reaction, and therefore provides criteria for the comparison of analogous enzymes. GBH of *P. putida* is inhibited by agmatine but not by guanidinoacetate, 3-guanidinopropionate, L-2-amino-4-guanidinobutyrate and other guanidino compounds.[32] L-Aspartate is a competitive inhibitor of guanidinosuccinate amidinohydrolase.[23] A variety of compounds of several categories has been tested and found to act as inhibitors of enzymes purified from *Pseudomonas* sp. ATCC 14676, *P. aeruginosa* PAO1, and *Flavobacterium* GE-1. The results will be shown later and discussed from comparative view points.

(3) pH Optimum

All amidinohydrolases so far studied exhibit alkaline optimal pH. As shown in Table I, Mn^{2+}-dependent amidinohydrolases exhibit somewhat higher pH optima than those of Zn^{2+} or Co^{2+}-dependent enzymes. The pH optima of arginases and other Mn^{2+}-dependent enzymes are in a range from 9.0 to 10.0, while those of type II enzymes are in a range from 8.0 to 8.5. Whether the difference is due to the nature of the active sites of enzymes or to that of metal ions themselves has not been elucidated. Zn^{2+} can slightly activate the apoenzyme of *P. aeruginosa* GPH. Preliminary experiments in our laboratory with the apoenzyme preincubated with Zn^{2+} or Mn^{2+} showed that the enzyme exhibited the same pH optimum with either metal ion (unpublished observations). This suggests that the difference of pH optima between the two types is not due only to the different metals.

(4) Thiol Groups

Some bacterial amidinohydrolases have been shown to be SH enzymes. Creatine amidinohydrolase is completely inactivated by modification of one mole SH per subunit protein with PCMB.[18] GAH of *Pseudomonas* sp. ATCC 14676 contains 2 moles of SH per subunit, one of which is essential for enzyme activity.[43] The essential SH of the native GAH reacts with PCMB but not with DTBN or N-ethylmaleimide, and the other SH does not react with PCMB unless the enzyme is treated with SDS preliminarily. The presence of Mn^{2+} protects the apo-GAH from inactivation with PCMB, indicating that the active SH locates in the active site.[43] *Flavobacterium* GAH and *Pseudomonas* GPH are also PCMB-sensitive and DTNB-insensitive. In most cases, PCMB-treated enzyme is reactivated by the treatment with 2-mercaptoethanol, to an extent of 60–80 % of the original activity.[27,31,43] Though GAH of *Flavobacterium* GE-1 is sensitive to PCMB, GBH of the same organism is PCMB-insensitive, and DTNB, iodoacetate, or N-ethylmaleimide fail to inhibit the enzyme. One may suspect, from the observation that Mn^{2+} protects the apoenzyme of *Pseudomonas* GAH from inactivation with PCMB, that the apoenzyme of the PCMB-insensitive GBH might react with PCMB. For testing this, a more effective method or conditions for the dissociation of Zn^{2+} from the native enzyme must be found out. It has been reported that heavy class arginases of chicken liver and *Neurospora* are PCMB-sensitive, while a light class arginase of rat liver is PCMB-insensitive.[41]

(5) Other Catalytic Properties

A common feature of bacterial amidinohydrolases is the relatively high *Km* values for the substrates, as shown in Table I. Most of the values are in the order of 10 mM. The *Km* of rat liver arginase for L-arginine is 20–40 mM and the values of chicken and *Neurospora* arginases are considerably higher.[41] Specific activities of purified amidinohydrolases exhibit a great variety, ranging from about 10 to 630. The specific activity of GBH of *Pseudomonas* sp. ATCC 14676 is 4 times as high as that of GAH of the same organism. By contrast, the specific activities of the two analogous enzymes of *Flavobacterium* are similar. If the evolutionary relationships among these analogous amidinohydrolases be evidenced, the difference in specific activity and *Km* will be an interesting subject from the view point of "improvement" of enzyme.

COMPARISON WITH INHIBITORS

As described in the preceding sections, bacterial amidinohydrolases other than arginase can be classified into two types, and each of them contains similar enzymes with respect to molecular weights, subunit sizes, metal requirements, and pH optima. The resemblance of enzymes of the same type suggests a hypothesis that these enzymes have evolved from a common ancestor in spite of their difference in substrate specificity. It is generally accepted that a pattern of competitive inhibitors of an enzyme relates closely to geometry of the active site of the enzyme. Comparison of enzymes with respect to inhibitor susceptibility is, therefore, one of the methods for approaching this problem when small amounts of purified enzyme preparations are available. This section describes several categories of competitive inhibitors of some enzymes of type I and type II.

(1) Substrate Analogs and Product Analogs

Table II shows the effect of various ω-guanidino and ω-amino acids on bacterial amidinohydrolases. Various guanidino compounds, which are the analogs of substrates of these enzymes, and various amino acids, which are the product analogs, have been tested as inhibitors. For all enzymes of type I and type II so far tested, it was observed that no homologs of the substrate guanidino acid of an enzyme did not inhibit the enzyme. By contrast, some enzymes are inhibited by ω-amino acids; a normal reaction product and its homologs with respect to each enzyme. In some cases, the next higher homolog of the product of an enzyme was a more effective inhibitor than the product itself. The mode of inhibition of *P. aeruginosa* GPH by β-alanine and 4-aminobutyrate was shown to be competitive nature, with *Ki* values much lower than the *Km* for the substrate 3-guanidinopropionate.[27] 4-Aminobutyrate and 5-aminovalerate inhibited competitively *Flavobacterium* GBH with *Ki* values being approximately the same as the *Km* for 4-guanidinobutyrate.[30] From these observations, there seem to be no appreciably different features between both enzyme types regarding the effect of this sort of inhibitors. It can be concluded that enzymes of both types commonly show relatively high binding specificities for

Table II. Inhibition by Various Compounds

Compound (5 mM)	Inhibition, % (*Ki*, mM)				
	Pseudomonas sp. ATCC 14676		*P. aeruginosa* PAO1	*Flavobacterium* GE-1	
	GAH[28]	GBH[29]	GPH[27]	GAH[a]	GBH[30]
Guanidinoacetate	—	0	0	—	10
3-Guanidinopropionate	4	6	—	—	10
4-Guanidinobutyrate	0	—	0	—	—
5-Guanidinovalerate	22	−10	4	nt	10
Glycine	50(2.9)	3	nt	40	0
β-Alanine	11	9	35(4.7)	10[b]	8
4-Aminobutyrate	11	85	54(2.8)	19[b]	46(2.7)
5-Aminovalerate	2	31	12	20[b]	65(0.5)
6-Aminocaproate	2	31	12	16[b]	23
Acetate	4	0	26	12	16
Propionate	0	49(2.0)	79(1.3)	22	39(1.6)
n-Butyrate	35	76(0.5)	64(2.3)	33	66(0.5)
n-Valerate	0	45	12	11	47(1.2)
n-Caproate	13	37	nt	nt	nt
trans-Crotonate	−8	67	9	30	62(1.5)
Glycolate	40	0	10	4	−16
DL-Lactate	0	−6	64(2.3)	nt	nt
DL-2-Hydroxybutyrate	0	9	62(2.3)	0	11
DL-3-Hydroxybutyrate	0	3	9(230)	−10	4
4-Hydroxybutyrate	0	6	38(6.9)	−5	9

a) Unpublished data except for the values with *ω*-amino acids.[24]
b) The values obtained at a concentration of 10 mM.
nt: not tested.

such a compound that has a positively charged group at one end of molecule and a negatively charged one at the other end.

(2) Lower Fatty Acids and Their Derivatives

As shown in Table II, several lower fatty acids and related compounds have been found to inhibit *Pseudomonas* and *Flavobacterium* amidinohydrolases, though the data with *Pseudomonas* GAH have not yet been published. Among this sort of compounds, propionate is the most effective inhibitor of *Pseudomonas* GPH, while n-butyrate is the most effective inhibitor of GBH of both types. The inhibition is rather strong in every case when examined at a concentration of 5 mM. The mode of inhibition of GPH by propionate or n-butyrate is competitive; the *Ki* values for the two compounds are in the same order as those for *ω*-amino acids and significantly lower than the *Km* for 3-guanidinopropionate. The nature of inhibition of *Flavobacterium* GBH by propionate, n-butyrate, or n-valerate is also competitive; the *Ki* values for these compounds are comparable to the *Km* for the substrate. It is the case for every enzyme that the number of carbon atoms of a fatty acid that is most effective inhibitor for an enzyme increases with increase of that of the normal sub-

strate of the enzyme. GBH of both types are inhibited by *trans*-crotonate, while this compound does not inhibit GPH. On the other hand, GPH is specifically inhibited by DL-2-hydroxybutyrate, 4-hydroxybutyrate, and DL-lactate, whereas the two GBH are almost insensitive to these hydroxy acids. It may be noteworthy that only DL-3-hydroxybutyrate inhibits GPH very slightly with about 30–100 times as high of *Ki* as those for the other hydroxy acids. These observations suggest that the active site of GPH, a type I enzyme, is not only smaller than, but also somewhat different from that of type I GBH, as well as that of type II GBH. No effect of the hydroxy acid on GBH of both types suggest the presence of narrow or hydrophobic regions in the active sites, to which the carbon atoms 2 to 4 of 4-guanidinobutyrate bind.

(3) Thiol Compounds

Several thiol compounds strongly inhibit the two *Flavobacterium* amidinohydrolases as shown in Table III. These compounds do not inhibit *Pseudomonas* (type I) enzymes at all. *Flavobacterium* GBH is competitively inhibited by thioglycolate with a very low *Ki* value of 8.7 μM. Inhibition by other thiol compounds tested is also competitive in nature; the *Ki* values are 10-fold less than the *Km* for the substrate. These thiol compounds inhibit both GAH and GBH with similar patterns, in which thioglycolate is the most effective followed by 3-mercaptopropionate and 2,3-dithio-1-propanol. The competitive nature of the inhibition indicates that these compounds bind to the active sites of these enzymes. The fact that glycolate has almost no effect on these enzymes suggests that the thiol group of thioglycolate as well as other thiol compounds is essential for its affinity to the enzymes. The part of the active site of enzyme to which thiol compounds bind may be different from that to which lower fatty acids bind, because the structures of the effective thiol compounds are somewhat varied compared with that those of the effective lower fatty acids are very limited.

It may be a possible explanation that thiol compounds interact with enzyme-

Table III. Inhibition of *Flavobacterium* Amidinohydrolases by Thiol Compounds

Compound	Concentration (mM)	GAH[28]	GBH[28]	
		Inhibition (%)	Inhibition (%)	Ki (mM)
Thioglycolate	0.1	80	87	0.009
	0.5	95	97	
3-Mercaptopropionate	0.1	44	32	0.07
	0.5	79	63	
2-Mercaptoethanol	0.5	32	37	0.43
2,3-Dimercapto-1-propanol	0.5	43	30	0.36
Dithiothreitol	0.5	30	34	nd
Mercaptoethylamine	0.5	7	13	0.56
	5.0	47	63	
L-Cysteine	0.5	43	11	nd

nd: not determined.

bound zinc as thiol compounds form complexes with zinc in aqueous media.[46] Certain thiol compounds inhibit some zinc enzymes. For example, L-cysteine is a potent inhibitor of bovine carboxypeptidase A[47] and dithiothreitol and 2-mercaptoethanol are potent inhibitors of mouse asites tumor dipeptidase.[48] Similar inhibition is observed with other zinc enzymes; *Escherichia coli* B dipeptidase[49] and carboxypeptidase C of orange leaves.[50] The action of these compounds on zinc enzymes is generally regarded as removal of metals by chelation. The action of thiol compounds on type II amidinohydrolases, however, is probably not simple chelation. As far as tested, the mode of inhibition by thiol compounds is competitive with substrate and these compounds fail to accelerate the dissociation of enzyme-bound metals with *o*-phenanthroline. These observations suggest that thiol compounds interact with enzyme-bound metals but the complex formed does not dissociate from enzyme.

The specific inhibition of *Flavobacterium* amidinohydrolases by thiol compounds may not necessarily reflect the dissimilarity of these enzymes to type I enzymes in their structure, but this fact should be useful for screening tests of type I or type II enzymes with crude extracts.

(4) Miscellaneous Compounds

The enzymes compared in this section act toward ω-guanidino carboxylic acids and produce ω-amino acids. Each of these compounds possesses a a negative charge at one end of molecule and a positive charge at another end. As described above, some ω-amino acids and monocarboxylic acids are inhibitors of both type I and type II enzymes. On the other hand, aliphatic amines such as n-butylamine, n-propylamine, and 3-aminopropanol fail to inhibit any of these enzymes. Dicarboxylic acids such as succinic acid, adipic acid, and fumaric acid, as well as various guanidino compounds other than substrate analogs such as creatine, N-amidinoalanine, L-arginine, and D-arginine also exhibit no effect on these enzymes. The presence of a negative charge at an end of molecule is necessary for a molecule in binding to enzyme. The reason why dicarboxylic acids of about the same sizes as that of substrate can not bind to enzyme is not known.

COMPARISONS BY GEL ELECTROPHORESIS AND GEL CHROMATOGRAPHY

As described in the preceding sections, the three amidinohydrolases of type I share some molecular and enzymic properties in common, and this is true also for the two enzymes of type II. On the other hand the metal requirement and the range of molecular weights of type I enzymes differ significantly from those of type II enzymes. The marked similarity of the enzymes of the same type sggests that these enzymes are evolutionary homologous. Therefore, direct comparisons by the use of two or more enzymes at the same time have been performed for certifying the difference.[28]

In general, isozymes have similar molecular properties but do not exhibit serological cross reactions, and sometimes exhibit significantly different electric charges as

is the case of animal lactate dehydrogenases. Thus, with respect to enzymes which have similar but different enzymic function, it may be reasonable to expect that some of them differ from each other in molecular properties to an extent. The sequence homology among mammalian serine proteases has been well documented,[51] but there is an extent of variety of their molecular weights, for example, chymotrypsinogen A (M.W., 25,000)[52] and thrombin (M.W., 33,700).[53] Direct comparisons of some sets of bacterial amidinohydrolases by electrophoretic technics showed that some enzymes of the same type were indistinguishable on electrophoresis in the presence or absence of SDS. These observations, therefore, strongly suggest that such amidinohydrolases have diverged from a common ancestral enzyme, though the validity of this proposal can be tested only by comparisons of the primary structures of the proteins.

(1) Comparison of Type I Enzymes

The molecular weights of GPH of *P. aeruginosa* PAO1 (195,000–215,000)[27] and GBH of *Pseudomonas* sp. ATCC 14676 (180,000–186,000) obtained in separate experiments were very close. A slightly lower value (163,000) was obtained for GAH of the latter organism.[24] Several experiments were carried out with combinations of fresh preparations of these enzymes by use of electrophoretic and chromatographic technics.[28] The former two enzymes showed very close mobilities on electrophoresis in gels prepared according to Davis[55] containing 6 % or 9 % polyacrylamide. Fig. 2A shows a single protein band observed when a mixture of these two enzymes was applied. Considered in conjunction with the observations described by Hedrick and Smith[54] regarding the molecular weight determination with polyacrylamide gels, this result indicates that these two enzyme proteins are quite similar in molecular weight as well as electric charge. These enzymes exhibited closely resembled mobilities on SDS-polyacrylamide gel electrophoresis. Fig. 2B shows a single band observed after the two enzymes were subjected to SDS-electrophoresis on the same gel, indicating marked similarity of the subunit sizes. When a mixture

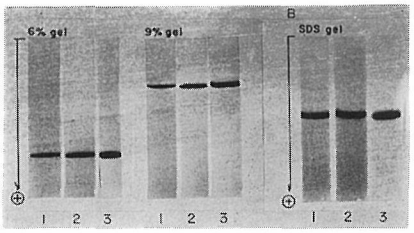

Fig. 2. Comparison of *Pseudomonas* amidinohydrolases by polyacrylamide gel disc electrophoresis.
A: 1, GPH (5.0 μg) of *P. aeruginosa* PAO1; 2, GBH (5.0 μg) of *Pseudomonas* sp. ATCC 14676; 3, GPH (5.0 μg) plus GBH (5.0 μg). Electrophoresis was performed according to Davis[55] with Tris-glycine pH 9.5 buffer system. B: 1, GPH (1.6 μg); 2, GBH (1.5 μg); 3, GPH (1.6 μg) plus GBH (1.5 μg). Each enzyme protein was incubated at 100°C for 5 min in the presence of 2% SDS and 2% 2-mercaptoethanol, and then electrophoresed according to Weber and Osborn.[67]

of these enzymes was applied to a Sephadex G-200 column, they eluted with an essentially overlapped elution profile. This observation also indicates their quite similar molecular weights. By contrast, GAH and GBH of *Pseudomonas* sp. ATCC 14676 did not give a single band on polyacrylamide electrophoresis and on SDS-electrophoresis, though they eluted at the same position when a mixture was subjected to gel chromatography. One may be impressed by the fact that GBH of *Pseudomonas* sp. ATCC 14676 shows a striking resemblance in molecular properties to GPH of another species of *Pseudomonas*, whereas exhibits slight difference with GBH from the same organism. The similarity of the two analogous enzymes of distinct bacterial species might be such an example that reflects the participation of gene transfer in bacterial acquisition of new enzymic function during the evolution. Although we have no information about the structural homology of these proteins, it should be considered that a single substitution of amino acid which occurs in a protein sometimes causes a significant change in electrophoretic mobility of the protein in SDS-gel electrophoresis.[58,59] Though the taxonomic relationship between *Pseudomonas* sp. ATCC 14676 and *P. aeruginosa* PAO1 has not been elucidated, some differences of the physiological properties have been detected. The former strain can not produce GPH, while the latter fails to produce GAH. GBH of the former strain is induced by L-arginine as well as 4-guanidinobutyrate, while L-arginine fails to induce GBH of the latter. The L-arginine degradation pathway of the latter organism has been well established[56] in which 4-guanidinobutyrate is not involved. Some observations in our laboratory suggested that L-arginine degradation in the former organism is the same type as that in *P. putida*[57] in which GBH catalyzes the fourth step.

(2) Comparison of Type II Enzymes

GAH and GBH of *Flavobacterium* GE-1 eluted with an almost coincident profile when a mixture of these enzymes were chromatographed on a Sephadex G-200 column,[28] indicating their quite similar molecular weights of about 240,000, though the previous separate experiments gave somewhat different values of 281,000[25] and 242,000,[30] respectively. In this experiment, the difference in the molecular weight of the two enzymes was estimated to be within 1 % of the molecular weights.[28] In the previous experiments,[25] a subunit molecular weight of 70,000 was obtained for GAH by SDS-gel electrophoresis after treatment with SDS at 50°C. When GBH was examined under similar conditions, two protein bands were observed; one corresponds to a molecular weight of 42,000 and the other 80,000. This indicated that these bands were the monomer and the dimer of the subunit. The monomer band increased with increase of the time of incubation with SDS (Fig. 3A). A similar profile was observed with GAH treated with SDS at 65°C (Fig. 3B). Both enzymes dissociated completely by the treatment at 100°C, and a mixture of the two SDS-treated enzymes gave a single band when applied to the same well of a SDS-gel slab and electrophoresed (Fig. 4). These observations indicate the close similitude of these enzymes; both enzymes dissociate to dimers on mild incubation with SDS, and the difference of the molecular weights of subunits can not be detected by SDS-

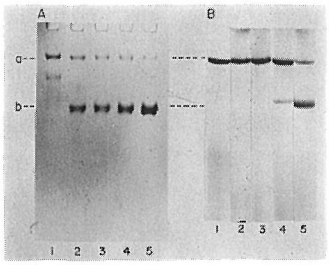

Fig. 3. SDS-polyacrylamide gel electrophoresis of *Flavobacterium* GE-1 amidinohydrolases treated with SDS under mild conditions.
A: GAH (3.1 μg) incubated in the presence of 2 % SDS and 2% 2-mercaptoethanol at 65°C for 0 min (1), 20 min (2), 40 min (3), 80 min (4), or 160 min (5), was applied to a well of a gel slab of 0.2-cm thickness. Other conditions were the same as those described in Fig. 2B. B: GBH was analyzed by the same procedure described above except that electrophoresis was performed with an apparatus for disc electrophoresis. Each sample contained 3.2 μg of enzyme protein incubated at 50°C in the presence of 2% SDS and 2% 2-mercaptoethanol for 10 min (1), 20 min (2), 60 min (3), 120 min (4), or 240 min (5). The mobilities of the protein bands corresponded to: a, 80,000 molecular weight; b, 42,000 molecular weight.

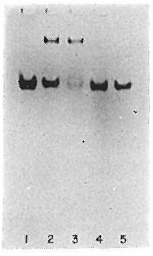

Fig. 4. Comparison of *Flavobacterium* GE-1 aminidinohydrolases by SDS-polyacrylamide gel electrophoresis.
The conditions for electrophoresis were the same as those described in Fig. 3A. The enzymes and the conditions for SDS-treatment were: 1, GAH (3.1 μg) plus GBH (3.2 μg), at 100°C for 5 min; 2, GAH (3.1 μg) plus GBH (3.2 μg), at 65°C for 80 min; 3, GAH (3.1 μg), at 65°C for 80 min; 4, GAH (3.1 μg), at 100°C for 5 min; 5, GBH (3.2 μg), at 100°C for 5 min.

electrophoresis. These facts strongly suggest that the two analogous amidinohydrolases of *Flavobacterium* GE-1 have evolved from an ancestral enzyme to exhibit clearly divergent substrate specificities.

CONCLUDING REMARKS

Bacterial amidinohydrolases which act toward ω-guanidino acids are all inducible enzymes and exhibit high substrate specificities. Some sets of these enzymes, purified to homogeneity, have proved to be closely related. Thus these enzymes provide additional raw materials for examining the idea that a new metabolic pathway has evolved by patchwork assembly of catalysts with low activity for the new reactions followed by gene duplication and enzyme "improvement."[60,61] Ornston

and his co-workers showed that the two sets of enzymes which catalyze the corresponding reactions in the two convergent parts of the β-ketoadipate pathway in *Pseudomonas putida* were similar,[62-64] and recently reported the partial amino acid sequences of some enzymes of the pathway indicating the striking homology among enzymes of *Pseudomonas* and *Acinetobacter*.[65] These observations may have great correlativity with the concepts of evolution of bacterial enzymes and bacterial phylogeny. The examinations of the analogous bacterial amidinohydrolases by peptide mapping technics, a micro-method deviced by Cleaveland *et al.*[66] for example, may provide information about the structural homology among these enzymes.

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