Arginyl Residues: Anion Recognition Sites in Enzymes

Abstract. *Chemical modification with 2,3-butanedione in borate buffer indicates that nine of ten glycolytic enzymes studied contain arginyl residues at their active sites. Fructose-1,6-diphosphatase also has arginines at its binding site for the allosteric inhibitor, adenosine monophosphate. These and other data suggest that, as a general rule, enzymes acting on anionic substrates or cofactors will probably contain arginyl residues as components of their ligand binding sites. This could account in part for the relatively infrequent occurrence of arginine in proteins.*

Arginyl residues can serve as positively charged recognition sites for negatively charged substrates and anionic cofactors in enzyme active sites. Reports from this laboratory (*1*) have shown that arginyl residues participate in binding the carboxyl group of substrates to carboxypeptidase A and aspartate aminotransferase; phosphate ester substrates to alkaline phosphatase and muscle aldolase; reduced nicotinamide adenine dinucleotide (NADH) to the alcohol dehydrogenases from horse liver, human liver, and yeast; adenosine triphosphate (ATP) to creatine kinase, glutamine synthetase, and carbamyl phosphate synthetase; and RNA template to the RNA-dependent DNA polymerases from type C RNA viruses.

Workers at other laboratories have identified at least 15 more enzymes that employ arginyl residues to interact with negatively charged phosphate or carboxylate moieties of substrates or cofactors (*2*). In several instances this has been confirmed by x-ray crystallography.

We have now examined the role of arginyl residues in the enzymes of the glycolytic pathway. This central metabolic process is especially interesting because each step involves phosphorylated substrates or coenzymes, or both. Moreover, all of the constituent enzymes have been or are currently the subject of both primary and three-dimensional structural analysis. Four of the glycolytic enzymes—alcohol dehydrogenase, aldolase, lactate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase—contain essential arginyl residues (*3*). Our present results indicate that nine of ten additional enzymes in this pathway also contain essential arginyl residues as indicated by chemical modification with butanedione in borate buffer, a system known to be highly sensitive for the modification of arginine (*4*).

All enzymes and substrates were obtained from either Sigma or Boehringer Mannheim. Enzymes were assayed by standard procedures as suggested by the supplier. Chemical modifications were carried out as described in Fig. 1 and Table 1.

Exposure to 20 m*M* butanedione in 50 m*M* borate, *p*H 8.3, rapidly inactivates eight of the nine glycolytic enzymes listed in Table 1, with half-inactivation times ranging from 1 minute for phosphoglycerate mutase to 21 minutes for phosphoglucose isomerase. In each of these experiments, borate enhanced the rate of inactivation from twofold, in the case of hexokinase, to 43-fold for phosphoglucose isomerase, relative to the half-inactivation times obtained in 50 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (Hepes) buffer.

It is interesting to compare phosphoglucose isomerase and triosephosphate isomerase, enzymes that catalyze analogous reactions. The former is inactivated at a rate that is slower than, but nevertheless comparable to, the other glycolytic enzymes. The latter is hardly inactivated at all by butanedione and borate. This is consistent with x-ray crystallographic studies on triosephosphate isomerase that reveal no arginyl residues in the region of the enzyme thought to encompass its active site (*5*).

The known specificity of butanedione (*1, 3, 4*) and the observed enhancement by borate indicate that the inactivation is due to modification of essential arginyl residues. Amino acid analysis and other direct means to verify the modification of arginine have not yet been employed. Residues other than or in addition to arginine might well be modified in some of the enzymes studied. Lysyl modification, for example, is known to inactivate both 3-phosphoglycerate kinase (*6*) and triosephosphate isomerase (*7*), but the activity of the latter enzyme is hardly affected by butanedione. It seems likely that the present observations are due to arginine modification.

Fructose-1,6-diphosphatase is one of two enzymes governing the control point of glycolysis, that is, the interconversion of fructose-6-phosphate and fructose-1,6-diphosphate. This allosteric enzyme has a catalytic site that binds fructose-1,6-diphosphate and a regulatory site sensitive to adenosine monophosphate (AMP) (*8*). Of the various fructose-1,6-diphosphatases, that from rabbit muscle

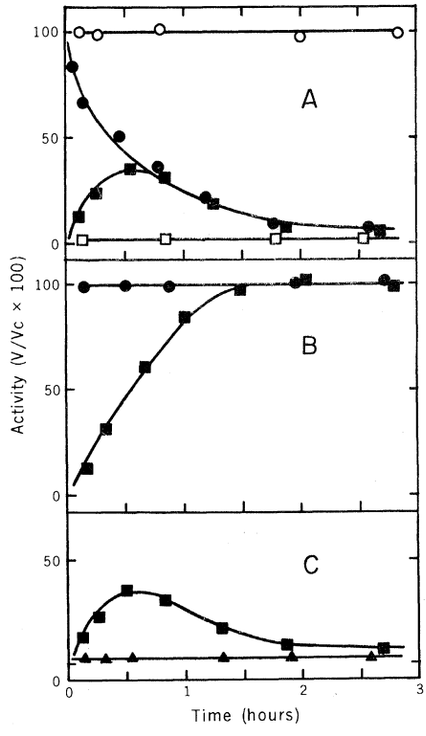

Fig. 1. Modification of rabbit muscle fructose-1,6-diphosphatase by butanedione and borate. Enzyme activity was determined spectrophotometrically at 25°C following the reduction of nicotinamide adenine dinucleotide phosphate (NADP) in the presence of excess phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. The assay mixture (1.0 ml) contained 40 m*M* tris chloride, *p*H 7.5, 5 m*M* $MgCl_2$, 0.1 m*M* fructose-1,6-diphosphate, and 0.1 m*M* NADP. The inhibition of AMP was measured by including 10 μM AMP in the otherwise identical assay mixture. Fructose-1,6-diphosphatase was modified at 25°C with 10 m*M* butanedione in 50 m*M* borate, *p*H 8.3. Portions were withdrawn at specific times and assayed for enzyme activity, both in the presence and in the absence of AMP, and residual activity is expressed as the ratio of the activity of the modified enzyme, *V*, to that of the control as determined by assaying in the absence of AMP, *Vc*, multiplied by 100. (A) Fructose-1,6-diphosphatase modification by butanedione was followed by assaying in the absence (●) or presence (■) of AMP. Also shown is the activity of the control, subjected to identical conditions but in the absence of butanedione, and assayed in the absence (○) or presence (□) of AMP. (B) Modification by butanedione was carried out in the presence of 1 m*M* fructose-1,6-diphosphate, and followed by assaying in the absence (●) or presence (■) of AMP. (C) Modification by butanedione was carried out in the absence (■) or presence (▲) of 10 μM AMP in the modification solution and followed by assaying in the presence of AMP. The assay without AMP could not be performed on this modification because, in the process of dilution into the assay mixture, sufficient AMP was introduced to reduce significantly the measured activity.

is by far the most sensitive to AMP inhibition. When assayed in the presence of 10 μM AMP, it exhibits only 3 percent of the activity obtained in the absence of AMP (Fig. 1A). Modification of fructose-1,6-diphosphatase with 10 mM butanedione in 50 mM borate, pH 8.3, progressively decreases activity as determined by the standard assay without AMP, with an inactivation half-time of 27 minutes. However, if the same modification is followed by assaying in the presence of 10 μM AMP, activity initially increases 13-fold. After 30 minutes activity in the presence or absence of AMP is the same (Fig. 1A). Thus, two processes can be differentiated. The first is modification of essential arginyl residues at the regulatory (AMP) site rendering the enzyme insensitive to AMP inhibition. The second is modification of arginyl residues at the catalytic site, with a concomitant loss of enzyme activity.

Protection experiments support this view. Modification in the presence of 1 mM fructose-1,6-diphosphate results in complete retention of activity in the standard assay without AMP. However, if this modification is followed by assaying in the presence of 10 μM AMP, activity increases rapidly for 75 minutes at which time it is identical with that observed in the absence of AMP (Fig. 1B). Thus, modification in the presence of fructose-1,6-diphosphate leads to complete loss of AMP inhibition but complete retention of catalytic activity. Finally, modification in the presence of 10 μM AMP does not affect AMP inhibition (Fig. 1C), indicating that AMP can fully protect against modification of arginyl residues at the regulatory site. Thus, rabbit muscle fructose-1,6-diphosphatase has essential arginyl residues at both its regulatory and catalytic sites. According to a recent report, the monovalent cation activation of fructose-1,6-diphosphatase from pig kidney also involves essential arginyl residues (*8*). It is unlikely that arginyl residues would be directly involved in cation binding, but obviously the phenomenon of monovalent cation activation is complex.

It has long been recognized that all of the intermediates in the glycolytic pathway are phosphoric acid esters. The phosphate group, in addition to being required for generating ATP from adenosine diphosphate (ADP), plays an important role in preventing metabolites from diffusing across cell membranes. The observation that all but one of the glycolytic enzymes contain essential arginyl residues plus similar findings for other enzymes further defines the role of phosphate in biological metabolites. The relative scarcity of arginyl residues in proteins, including enzymes, where they occur far less frequently than expected from the number of available arginine codons, has been thought to reflect either genetic pressure restricting arginine to certain important biological functions (*9*), or the late appearance of arginine in proteins during evolution (*10*).

Table 1. Inactivation of glycolytic enzymes by butanedione. Modifications were carried out at 25°C with 20 mM butanedione in either 50 mM borate or 50 mM Hepes, pH 8.3. Portions were withdrawn at specific times and assayed for enzymatic activity and compared to controls which were subjected to the same conditions except that no butanedione was added. Times for 50 percent inactivation, $T_{1/2}$ values, were determined from semilogarithmic graphs in which residual activity was plotted against time; ND, not determined.

Enzyme	E.C. number	$T_{1/2}$ (minutes) for inactivation in	
		50 mM borate	50 mM Hepes
Hexokinase	2.7.1.1	3.4	6.0
Phosphoglucose isomerase	5.3.1.9	21.0	900.0
Fructose-6-phosphate kinase	2.7.1.11	3.6	ND
Triosephosphate isomerase	5.3.1.1	780.0	ND
3-Phosphoglycerate kinase	2.7.2.3	6.8	42.3
Phosphoglycerate mutase	2.7.5.3	1.0	2.1
Enolase	4.2.1.11	3.0	ND
Pyruvate kinase	2.7.1.40	12.7	29.0
Phosphoglucomutase	2.7.5.1	3.7	14.1

One major biological function of arginine might be to interact with phosphorylated metabolites. Studies have shown that the guanidinium group is ideally suited for such interaction with phosphate-containing substances by virtue of its planar structure and its ability to form multiple hydrogen bonds with the phosphate moiety (*11*). Lysyl residues might also have been selected to serve this function and, indeed, are known to be important in a number of enzymes acting on phosphorylated substrates. However, because of resonance stabilization, the guanidinium group is a poor proton donor ($pK_a > 12$) and, hence, would probably not function as a general acid catalyst for the hydrolysis of the phosphorylated intermediates. This would further ensure maximum utilization of substrate phosphate for the synthesis of ATP and thereby contribute to the overall efficiency of glycolysis. Thus, the phosphorylation of glycolytic metabolites confers an extra degree of specificity in the proper interaction between the metabolites and enzyme arginyl residues. The selection of arginyl residues for this function minimizes nonspecific hydrolysis and optimizes the metabolic process.

In the energetics of living systems, ATP occupies a critical position. It is not known at what stage of evolution this unique conductor of chemical energy first emerged. If it was prior to the acquisition of a macromolecular coding system, then evolutionary pressure could have restricted the role of arginine in proteins to anion, particularly phosphate, binding. Optimal binding specificity could then be achieved by limiting the number of arginyl residues in proteins. If the ATP system emerged subsequent to the coding system, the code might have required amendment to include arginine, perhaps by adopting the transfer RNA of some other amino acid, for example, ornithine (*10*), that is less suitable for binding phosphate. Late appearance of arginine codons could then account for the relative scarcity of this residue in proteins. Although present investigations do not favor either alternative, they clearly emphasize an important generality, that arginyl residues consititute positively charged binding sites for enzymes acting on anionic substrates.

Finally, it is of interest that in instances where stoichiometry has been established (*1–4*) arginine modification generally appears to be quite selective. A comparison of the rate of modification of the essential arginyl residue of creatine kinase, for example, with that of arginine itself revealed that the arginyl residue in the enzyme reacted 10 to 15 times faster than the free amino acid (*1*). This hyperreactivity, coupled with a decreased reactivity of all the other arginyl residues in the enzyme, probably reflects a structural environment that enhances the binding capabilities of the active site arginyl residue thereby further potentiating catalysis.

J. F. RIORDAN
Biophysics Research Laboratory,
Department of Biological Chemistry,
Harvard Medical School,
Boston, Massachusetts 02115
K. D. MCELVANY
C. L. BORDERS, JR.
Department of Chemistry,
College of Wooster,
Wooster, Ohio 44691

References and Notes

1. C. L. Borders, Jr., and J. F. Riordan, *Biochemistry* **14**, 4699 (1975); ——, D. S. Auld, *Biochem. Biophys. Res. Commun.* **66**, 490 (1975), and additional references therein.
2. L. Patthy and E. L. Smith, *J. Biol. Chem.* **250**, 565 (1975), and references therein.
3. L. G. Lange, III, J. F. Riordan, B. L. Vallee, *Biochemistry* **13**, 4361 (1974); R. R. Lobb *et al.*, *FEBS Lett.* **54**, 70 (1975); P. Yang and G. W. Schwert, *Biochemistry* **11**, 2218 (1972); N. K. Nagradova and R. A. Asryants, *Biochim. Biophys. Acta* **386**, 365 (1975).
4. J. F. Riordon, *Biochemistry* **12**, 3915 (1973).
5. D. W. Banner, A. C. Bloomer, G. A. Petsko, D. C. Phillips, C. I. Pogson, I. A. Wilson, P. H. Corran, A. J. Furth, J. D. Milman, R. E. Offord, J. D. Priddle, S. G. Waley, *Nature (London)* **255**, 609 (1975).
6. F. S. Markland, A. D. E. Bacharach, B. H. Weber, T. C. O'Grady, G. C. Saunders, N. Umemura, *J. Biol. Chem.* **250**, 1301 (1975).
7. P. M. Burton and S. G. Waley, *Biochem. J.* **104**, 3P (1967).
8. F. Marcus, *Biochemistry* **14**, 3916 (1975).
9. M. Wallis, *Biochem. Biophys. Res. Commun.* **56**, 711 (1974).
10. T. H. Jukes, *ibid.* **53**, 709 (1973).
11. F. A. Cotton, E. E. Hazen, Jr., V. W. Day, S. Larsen, J. G. Norman, Jr., S. T. K. Wong, K. H. Johnson, *J. Am. Chem. Soc.* **95**, 2367 (1973).

12. Supported by grant-in-aid GM-15003 from NIH (to B. L. Vallee, principal investigator), and by a Cottrell College Science grant from Research Corporation (C.L.B.). We thank B. L. Vallee and L. G. Lange, III, for valuable discussions and assistance. C.L.B. was at the Biophysics Research Laboratory on research leave from the College of Wooster during most of this work.

20 August 1976; revised 8 October 1976

Chemosensory Tracking of Scent Trails by the Planktonic Shrimp *Acetes sibogae australis*

Abstract. *In the laboratory, planktonic shrimps* (Acetes sibogae) *precisely follow scent trails of food or paper soaked in meat extract,* L-*alanine,* L-*leucine, and* L-*methionine. In the ocean,* Acetes *may be able to follow scent trails as far as 20 meters to catch falling food. This demonstrates precise trail-following by pelagic animals.*

The importance of chemosensitivity in the marine environment has been emphasized, and highly sensitive chemoreceptive abilities have been described for benthic and pelagic animals (*1*); but almost no data are available for plankton. In the laboratory, copepods "search" for food or females (*2*); chemoreceptors have been described for several planktonic crustaceans (*3, 4*). The planktonic sergestid shrimp *Acetes sibogae australis* Colefax becomes increasingly active when stimulated by food scents, but when trials are run in appropriately designed aquariums this generalized searching behavior transforms into the most precise chemosensory trail-following response yet observed for any free-swimming aquatic animal including fishes (*1, 5*). This suggests that chemical perception in free-swimming organisms may be extremely effective ecologically and deserves additional attention.

Acetes sibogae australis were collected at night with light traps suspended about 2 m deep in the harbor off Townsville, Queensland, Australia, as well as in shallow water off the beach. In each unaerated aquarium (15 by 22 by 60 cm), 8 to 12 shrimps were kept and fed *Artemia*; water was changed every 2 days. Experiments were conducted in these aquariums. Each aquarium was illuminated from both sides by fluorescent light; the backs and bottoms of the tanks were painted black to make the transparent shrimps more visible. The fluorescent green dye used in the experiments became brightly visible under these conditions. The front pane was marked at 5-cm intervals to estimate swimming speed and distance. Experimental food stimuli consisted of diced banana prawns (*Penaeus merguiensis*) or freshly killed and crushed *Acetes* soaked in fluorescein dye and dropped into the quiet water of the tall aquariums. These sank slowly to the bottom, leaving erratic but unbroken thin trails of highly visible dye in the water. Small (2 mm^2) pieces of dyed blotting paper soaked with meat extract or amino acids also served as test stimuli. The shrimps' sensitivity to chemical trails decreased with successive tests, and the water in the tanks became murky from the dye, so that the water always was changed after four to five tests. In each series of tests, uncontaminated blotting paper soaked in dye was presented as a control at the start and end of the experiment.

In the absence of food stimuli, the shrimps swam in horizontal linear paths back and forth across the width of the aquarium (22 cm) at an average speed of 1.9 cm/sec. When dyed food was dropped into the aquarium, *Acetes* never deviated from the linear paths to avoid or seek out the trail. But, when a "patrolling" shrimp did by chance contact a trail head-on, its swimming behavior immediately changed to one of two distinctly different modes. The shrimp either would initiate a rapid horizontal circular "search pattern" in the vicinity of the trail or it would sharply reorient the body axis to the vertical trail and rapidly track the exact, but often erratic, path left by the falling food, swimming at approximately three times the normal speed (Fig. 1). (Average speed of 19 animals following food trails was 5.6 cm/sec.) Food particles and blotting paper sank to the bottom of the 60-cm-high aquarium in about 30 seconds, and, since *Acetes* swam down the twisting trail at about three times this speed, they often caught the particles in mid-water. The *Acetes* then ate the food but would discard impregnated blotting paper. If an *Acetes* lost the trail when the trail recurved at a sharply acute angle, it then swam a circular search pattern, usually relocated the trail, and again tracked the falling food. Some animals lost and relocated a trail five successive times as they traced it down through the water column.

Vision did not appear to play a role in trail-following. Both undyed meat and undyed blotting paper soaked in Millipore-filtered prawn juice elicited trail-following of the same precision and rapidity as did the visible scent trails. Furthermore, no shrimp among the thousands we watched appeared to deviate from its normal path to enter a visible dye trail with or without scent. Animals that had repeatedly responded to contact with visible scent trails never responded to contact with the visible but chemically clean control trails, which terminated as well as preceded each experiment.

An initial experiment recorded the behavioral response of *A. sibogae australis* to dyed pieces of shrimp. Of 63 animals that contacted the food trails, 54 responded; 39 of these followed the trail for at least 10 cm. These figures imply that the trail-following response was somewhat equivocal, but these data belie the regularity with which hundreds of *Acetes*, collected during a 3-month period, displayed the response. Experimental factors, such as chemical contamination from previous tests in the same water or reduced receptiveness of individual sergestids probably accounted for lower levels of response in the initial experiment. In a second experiment, the shrimps responded positively in 100 percent of their contacts with the trails, and in all experiments (with meat or meat extract) every trail contacted always elicited at least one tracking response.

Acetes sibogae australis rarely swam upward when following a vertical trail. Of the 54 animals responding in the initial experiment, one swam up a trail about 5 cm, then reversed and swam downward. In over 1000 subsequent tests, *Acetes* swam up a trail only six times. To determine whether *A. sibogae australis* generally swims down or follows a chemical gradient, we lowered dyed meat tied to a string near one side