Stoll, E., & Domb, C. (1978) *J. Phys. A: Math., Nucl. Gen. 11*, L57-L61.
Stoll, E., Binder, K., & Schneider, T. (1972) *Phys. Rev. B 6*, 2777-2780.
Sur, A., Lebowitz, J. L., Marro, J., Kalos, M. H., & Kirkpatrick, S. (1976) *J. Stat. Phys. 15*, 345-353.
Sykes, M. F., & Essam, J. F. (1963) *Phys. Rev. Lett. 10*, 3-6.
Taylor, R. B., Duffus, W. P. H., Raff, M. D., & DePetris, S. (1971) *Nature (London) 233*, 225-230.
Thompson, T. E. (1978) in *Molecular Specialization and Symmetry in Membrane Function* (Solomon, A. K., & Karnousky, M., Eds.) pp 78-98, Harvard University Press, Cambridge, MA.
Thompson, T. E., & Huang, C. (1978) in *Physiology of Membrane Disorders* (Andreoli, T. E., Hoffman, J. F., & Fanestil, D. D., Eds.) pp 27-48, Plenum Press, New York.
Van Dijck, P. W. M., de Kruijff, B., Verkleij, A. J., Van Deenen, L. L. M., & de Gier, J. (1978) *Biochim. Biophys. Acta 512*, 84-96.
Von Dreele, P. H. (1978) *Biochemistry 17*, 3939-3943.
Wallace, B., & Engelman, D. (1978) *Biochim. Biophys. Acta 508*, 431-449.

Identification of an Arginine Important for Enzymatic Activity within the Covalent Structure of Yeast Inorganic Pyrophosphatase†

M. W. Bond,‡ N. Y. Chiu,§ and B. S. Cooperman*

ABSTRACT: Previously we presented evidence for an essential arginine involved in binding inorganic pyrophosphate during catalysis by yeast inorganic pyrophosphatase [Cooperman, B. S., & Chiu, N. Y. (1973b) *Biochemistry 12*, 1676]. In the present work we show this residue to be arginine-77. Arginine-77 reacts with [^{14}C]phenylglyoxal considerably faster than the other five arginine residues in the enzyme subunit, and its reaction with phenylglyoxal is selectively blocked in the presence of the competitive inhibitor calcium pyrophosphate. Our procedure leading to the identification of Arg-77 utilizes the following steps: CNBr cleavage, digestion with *Staphylococcus aureus* V8 protease and with pepsin, and peptide mapping. All of these steps are performed below pH 5, a restriction imposed by the lability of the phenylglyoxal-arginine adduct at neutral pH. In related work, we find the model compound N^{α}-acetyl(diphenylglyoxal)arginine to hydrolyze 10 times more slowly at pH 4 than at pH 7. The high yields of derivatized peptides obtained in this work suggest the potential general utility of our procedure for locating arginine residues derivatized with phenylglyoxal within the covalent structure of proteins.

Despite their widespread distribution and central importance to cellular metabolism, phosphoryl-transfer enzymes remain incompletely understood with respect to their detailed mechanisms, especially when compared with what is known about more well studied enzymes, such as the serine proteases (Blow, 1976; Kraut, 1977). This situation is in the process of changing. In recent years, high-resolution X-ray structures have been reported for adenylate kinase (Pai et al., 1977), hexokinase (Anderson et al., 1978), and pyruvate kinase (Levine et al., 1978), and this new information, when coupled with the large body of information available from studies of these enzymes in solution, should lead to the formulation of more detailed models of enzyme mechanism. Yeast inorganic pyrophosphatase, EC 3.6.1.1 (PPase),[1] is another phosphoryl-transfer enzyme for which information is rapidly accumulating. The covalent structure has been determined (Cohen et al., 1978), two research groups have reported low-resolution crystal structures (Bunick et al., 1978; Makhaldiani et al., 1978), and a high-resolution structure determination is underway.[2] In addition, a fair amount is known about the stoichiometries and affinities of divalent metal ion, pyrophosphate and phosphate binding (Ridlington & Butler, 1972; Cooperman & Chiu, 1973a; Rapoport et al., 1973; Hamm & Cooperman, 1978; D. J. Hamm, B. Springs, and B. S. Cooperman, unpublished experiments), about the kinetic and chemical mechanisms (Rapoport et al., 1972; Moe & Butler, 1972; Konsowitz & Cooperman, 1976; Hackney & Boyer, 1978), about the orientation of metal ions and phosphate ligands at the active site (Hamm & Cooperman, 1978), and about the identity and roles of essential amino acid residues (Cooperman & Chiu, 1973b; Heitmann & Uhlig, 1974).

We and others (Cooperman & Chiu, 1973b; Heitmann & Uhlig, 1974) have previously shown that the arginine-specific reagent phenylglyoxal (PhGx) (Takahashi, 1968, 1977a,b) fully inactivates PPase in a pseudo-first-order process, that the rate constant for inactivation can be substantially decreased in the presence of competitive inhibitors, and that the modified PPase loses the ability to bind such competitive inhibitors. These results were taken as evidence for an essential arginine at the active site of PPase and are consistent with the known

† From the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received May 17, 1979.* This work was supported by a research grant from the National Institutes of Health (AM-13212).

‡ National Institutes of Health Predoctoral T32 GM-07229 Trainee (1976-1978). Present address: Division of Biology, California Institute of Technology, Pasadena, CA 91125.
§ Present address: Department of Chemistry, National Tsing Hua University, Hsinchu, Taiwan, Republic of China.

[1] Abbreviations used: BAWP, butanol-acetic acid-pyridine-water (15:3:10:12 v/v/v/v); NAcArg(PhGx)$_2$, N^{α}-acetyl(diphenylglyoxal)arginine; Arg(PhGx)$_2$, (diphenylglyoxal)arginine; PPase, yeast inorganic pyrophosphatase; PhGx, phenylglyoxal; SAV8P, *Staphylococcus aureus* V8 protease.

[2] D. Voet (personal communication).

0006-2960/80/0419-0094$01.00/0

ability of guanidinium salts to form complexes with phosphates (Cotton et al., 1974) and the common finding that enzymes with anionic substrates can often be inactivated by arginine-specific reagents (Riordan et al., 1977).

In the work described below, we obtained evidence that modification of a single arginine residue results in complete or almost complete inactivation of PPase and have identified, through the use of [^{14}C]PhGx, Arg-77 as the residue of interest. The importance of this result for PPase is that it will allow a first guess to be made as to the location of the active site in the forthcoming high-resolution X-ray structure. In the course of this work we have also measured the rate of hydrolysis of the NAcArg(PhGx)$_2$ adduct as a function of pH. The adduct was found to be considerably more labile at neutral than at low pH, which prompted us to develop an identification procedure in which all chemical fragmentation and proteolysis steps are carried out below pH 5.

Experimental Section

Materials

PPase (570–720 IU/mg) was prepared from baker's yeast as previously described (Cooperman et al., 1973). After the final column step it was precipitated with ammonium sulfate and stored at −20 °C. PhGx (Aldrich) was twice recrystallized from water before use. N^{α}-Acetyl(diphenylglyoxal)arginine, NAcArg(PhGx)$_2$, was synthesized according to the method of Takahashi (1968) except that 2.0 equiv of PhGx was used. In our hands, chromatography of the reaction mixture on Sephadex G-10 (Pharmacia) led to a product contaminated with free PhGx. Satisfactory purification, as judged by ^{1}H NMR, TLC, and paper chromatography, was achieved by rechromatography on Biogel P-4 (100–200 mesh) (Bio-Rad Laboratories) with elution by acetic acid–propanol–water (2:10:88 v/v/v). Fractions containing the pure compound were pooled, lyophilized, and stored desiccated at −20 °C. [^{14}C]-PhGx was synthesized at a specific radioactivity (6 mCi/mmol) significantly higher than that previously reported (Takahashi, 1968; Weng et al., 1978) from [^{14}C]acetophenone (6 mCi/mmol, New England Nuclear) by microscale adaptation of the method of Riley & Gray (1947). It was shown to be radiochemically pure by TLC (silica gel) in $CHCl_3$ (PhGx, R_f 0.16; acetophenone, R_f 0.70) and petroleum ether (60–110 °C)–ethyl acetate (1:1 v/v) (PhGx, R_f 0.34; acetophenone, R_f 0.64) by using a Packard Model 7201 radiochromatogram scanner. [^{14}C]PhGx stored at −20 °C appears to be stable indefinitely. The crystals melt on heating to 80 °C, permitting aliquots to be taken readily.

Methods

Hydrolysis of NAcArg(PhGx)$_2$. Fresh stock solutions of NAcArg(PhGx)$_2$ in ethanol (~0.01 M) were prepared daily. Aliquots of the stock solution were diluted into quartz cuvettes containing the appropriate buffer, and absorbance at 250 nm was monitored as a function of time on a Beckman Kintrac VII or a Cary 15 spectrophotometer, thermostated at 25 °C. The results were plotted as log $(A_\infty - A_t)$ vs. time, where $A_\infty = 1.88\ A_{initial}$.

Analytical Methods. Acid hydrolyses were performed by placing samples containing 0.2–5 nmol of peptide in 2 mL of 6 N HCl and 1–5 μmol of phenol in an ignition tube. The samples were thoroughly evacuated, sealed, and hydrolyzed for 18–24 h at 105–110 °C. The resultant hydrolysates were analyzed for amino acids by automated ion-exchange chromatography on either a Durrum D-500 analyzer (courtesy of Dr. R. L. Heinrikson, Department of Biochemistry, University of Chicago) or a Durrum fluorescence analyzer kit (courtesy of Dr. R. A. Hogue-Angeletti, Department of Neuropathology, University of Pennsylvania). Amino-terminal analyses were performed by the dansyl chloride method of Gray (1972) as modified by Weiner et al. (1972). Fluorescamine (Fluram, Roche Diagnostics) was used to detect and quantitate small amounts (10–50 pmol) of protein (Udenfreund et al., 1972). Fluorescence was measured on a Perkin-Elmer MPF-4 fluorescence spectrometer with excitation at 390 nm and emission monitored at 475 nm. Measurements of radioactivity were made by using an Intertechnique SL 30 scintillation spectrometer. The scintillation cocktail was a 1:1 mixture of Triton X-100 (Sigma) and a toluene solution containing 5.0 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (Me_2POPOP) per L. All components of the toluene solution were obtained from Fisher.

PPase Modification by [^{14}C]PhGx. PPase, precipitated with ammonium sulfate, was redissolved in and dialyzed against the reaction buffer. PhGx was added at zero time. Aliquots taken at appropriate times were assayed for residual enzymatic activity by the pH stat method (Cooperman et al., 1973) and for [^{14}C]PhGx incorporation by two different procedures. In procedure A, aliquots were quenched by dilution into glacial acetic acid and PPase was precipitated with 10% trichloroacetic acid by using bovine serum albumin (1 mg/mL) as carrier. After thorough washing with 10% trichloroacetic acid, the pellet was redissolved in base and the radioactivity determined as described earlier (Brunswick & Cooperman, 1971). In procedure B, aliquots used to measure [^{14}C]PhGx incorporation were quenched by diluting 10-fold in 10% HCO_2H and freezing in dry ice–acetone. Excess PhGx was removed by two filtrations on Sephadex G-25 in 10% HCO_2H at 4 °C. The second procedure, though more laborious, gave lower background values and is preferred. Values of [^{14}C]PhGx incorporation per PPase subunit reported are based upon a subunit molecular weight of 32042 (Cohen et al., 1978). Subunit concentration was determined by using $A_{280}^{1\%} = 14.5$ (Kunitz, 1952) or by fluorescamine assay. PPase samples for the latter measurements were clarified by filtration through a glass wool plug in a Pasteur pipet.

Random Labeling of PPase-Derived Peptides with [^{14}C]-PhGx. Peptides to be derivatized were dissolved in a minimal amount of 0.2 M *N*-ethylmorpholine acetate, pH 8.2. Citraconic anhydride as a 10% acetone solution was added dropwise with stirring over a period of 1 h to a 100-fold excess over the amino groups while the pH was monitored and maintained between 8 and 9 with concentrated aqueous NaOH (Dixon & Perham, 1968; Habeeb & Atassi, 1970). One-half hour after the last addition of anhydride the solution was chromatographed on Sephadex G-75 (2.2 × 110 cm) in 0.5% ammonium bicarbonate. Peptide CNBr II (see Scheme I) eluted at 0.42 of the column volume; peptide SA IV (see Scheme I) eluted at 0.68 of the column volume. The peptide thus freed of excess reagent and byproducts was lyophilized and redissolved in a minimal amount of 0.2 M *N*-ethylmorpholine acetate, pH 7.0 (peptide concentration ~0.5 mM). [^{14}C]PhGx was added to a final concentration of 0.05–0.1 M. The incubation was carried out at 25 °C for the length of time necessary (approximately one half-life) to incorporate ~3 mol of PhGx per mol of CNBr II (which contains three Arg) or ~2 mol of PhGx per mol of SA IV (which contains two Arg; see Scheme I), assuming a second-order rate constant of 0.15 M^{-1} min^{-1} (Takahashi, 1968). Phenylglyoxylation could not be carried to completion since prolonged incubation resulted in formation of a precipitate. At the end of the incubation period, the reaction mixture was acidified with 6 M urea

Table I: pH Dependence of NAcArg(PhGx)$_2$ Hydrolysis, 25 °C

pH	$k_{obsd} \times 10^3$ (min^{-1})
4.0[a]	0.2
4.5[a]	0.5
5.0[b]	0.5
5.5[b]	0.7
6.0[c]	1.4
6.5[c]	2.0
7.0[d,e]	2.5

[a-e] Buffers utilized: [a] acetic acid-sodium acetate; [b] *N,N*-dimethylpiperazine-HCl; [c] 2-(*N*-morpholino)ethanesulfonate; [d] *N*-ethylmorpholine-HCl; [e] sodium phosphate. No major buffer effects were observed over the concentration range 0.05–0.20 M.

(deionized) in 10% HCO_2H and immediately chromatographed on either Sephadex G-25 (2 × 61 cm) in 10% HCO_2H or Sephadex G-50 (2.5 × 180 cm) in 10% HCO_2H–10% propanol. This chromatography step serves to regenerate the free amino groups. Fractions containing the derivatized peptide were pooled, lyophilized, and stored at −87 °C.

Procedures used in CNBr cleavage of PPase and in SAV8P and pepsin proteolysis of PPase fragments are described in detail in the relevant figure legends.

Results

Studies on the Hydrolysis of NAcArg(PhGx)$_2$. Although it was shown by Takahashi (1968) that NAcArg(PhGx)$_2$ is less stable at neutral pH than in acid, there is very little published quantitative data concerning the rate of hydrolysis of the diadduct. The importance of such information for our ultimate purpose of isolating PhGx-derivatized peptides from PhGx-modified PPase led us to measure the hydrolysis rate as a function of pH. Since the ultraviolet absorbance maxima (~250 nm) and molar extinction coefficients which we determined for NAcArg(PhGx)$_2$ (1.2×10^4, our measurement; 1.1×10^4, Takahashi (1977b)] and PhGx (1.13×10^4) are very similar, the release of 2 mol of PhGx on hydrolysis of NAcArg(PhGx)$_2$ is accompanied by a large increase in A_{250}, which provides a convenient spectrophotometric method for determining rate constants for hydrolysis. Rate studies at 25 °C were performed in the pH range 4–7 and the approximate rate constants determined are listed in Table I. From a practical point of view, these results indicate the desirability of using low pH procedures to isolate Arg(PhGx)$_2$ diadducts, below pH 4 if possible but certainly not above pH 5.5.

The sharp rise in hydrolysis rate between pH 5.5 and 6.0 may arise from dissociation of a proton from the diadduct leading to formation of a more labile form and is consistent with a small, pH-dependent shift in the λ_{max} for NAcArg-(PhGx)$_2$, which decreases from 253 to 249 nm on increasing pH, with an apparent pK_a of 5.6–5.7 (data not shown). The kethoxal adduct of NAcArg has a similar pK_a value (Iijima et al., 1977). The molar extinction coefficients of NAcArg-(PhGx)$_2$ and PhGx are unchanged between pH 4 and 8. At pH values 4–6 A_{250} increases 1.9-fold over the course of hydrolysis, as expected for full release of PhGx. No evidence was found for accumulation of a monoadduct species during hydrolysis. At pH values ≥7 the hydrolysis of NAcArg-(PhGx)$_2$ is more complex. For example, at pH 8 (Figure 1) the ultraviolet spectral change is biphasic with time. The initial, expected increase of A_{250} is followed by a subsequent decrease accompanied by the appearance of a new absorbance peak at 230 nm. Suitable control experiments (data not shown) have established that PhGx itself is stable at pH 8 and does not give rise to such a peak. A model consistent with what we observe, although certainly not yet established, is shown in eq 1, in which the slowly formed species of unknown stoichiometry is responsible for the new peak.

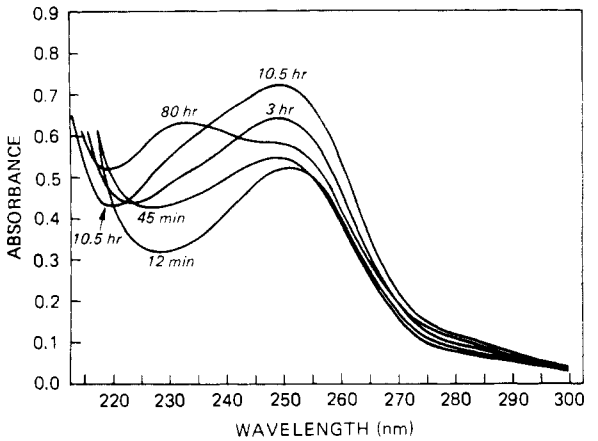


FIGURE 1: Ultraviolet spectral changes on hydrolysis of NAcArg-(PhGx)$_2$. Experimental conditions: 0.1 M sodium phosphate buffer, pH 8.0, 25 °C.

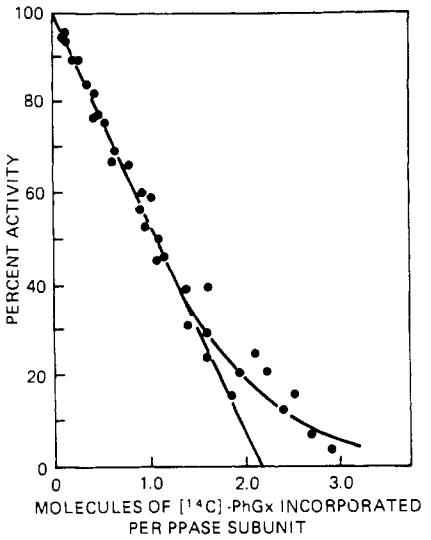


FIGURE 2: Correlation of [^{14}C]PhGx incorporation with PPase inactivation. PhGx incorporation was measured by procedure A. Experimental conditions: 0.08 M *N*-ethylmorpholine acetate (pH 7.0), 0.037 M PhGx, PPase (4 mg/mL), 35 °C.

$$\text{NAcArg(PhGx)}_2 \xrightarrow{\text{fast}} \text{NAcArg} + 2\text{PhGx} \xrightarrow{\text{slow}} (\text{NAcArg})_x(\text{PhGx})_y \quad (1)$$

Correlation of PhGx Inactivation of PPase and [^{14}C]PhGx Incorporation into PPase. We previously showed that treatment of PPase with PhGx resulted in the complete loss of enzymatic activity (<0.1%) in a process that displayed good pseudo-first-order kinetics to at least 3 to 4 half-lives, with a rate constant some 5 times faster than that obtained on reaction of PhGx with *N*-acetylarginine (Cooperman & Chiu, 1973b). In Figure 2 we show a correlation of the loss of enzymatic activity with ^{14}C incorporation. Extrapolation of the initial, linear portion of the curve gives full inactivation for the incorporation of ~2 PhGx molecules/PPase subunit, corresponding to the modification of a single arginine. This result, coupled with our earlier finding of a single, pseudo-first-order process for inactivation, leads to the conclusion that full or almost full inactivation results from the modification of a single, highly reactive, arginine. The increase in [^{14}C]-PhGx incorporation at later stages of the reaction would be expected as a result of the modification of more slowly reacting

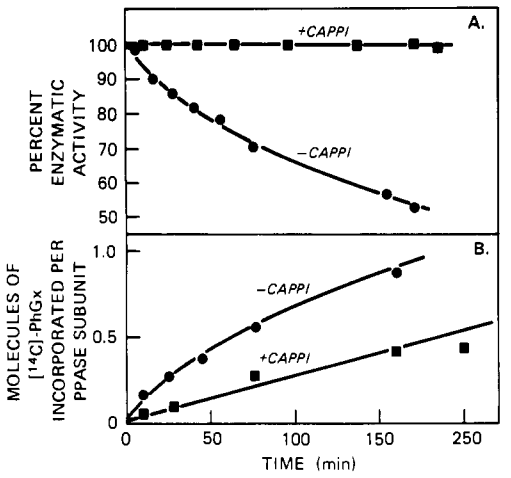
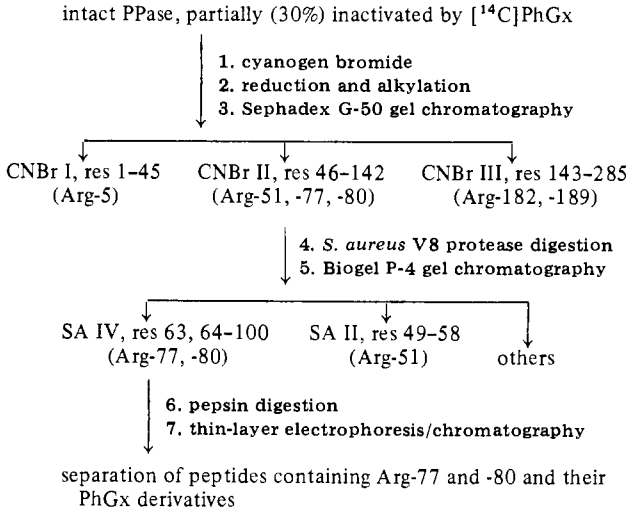


FIGURE 3: Modification of PPase by [^{14}C]PhGx in the presence and absence of Ca^{2+} (2 mM) and PP_i (1 mM). (A) Residual enzymatic activity in the absence (●) or presence (■) of $CaPP_i$. (B) [^{14}C]PhGx incorporation in the absence (●) or presence (■) of $CaPP_i$. PhGx incorporation was measured by procedure B. Experimental conditions: 0.2 M *N*-ethylmorpholine acetate (pH 7.0), 0.01 M PhGx, PPase (20 mg/mL), 25 °C.

Scheme I: Overall Scheme of Peptide Generation and Fractionation from Modified PPase



arginines. Such incorporation can at most have only a very minor effect on PPase inactivation.

$CaPP_i$ Protection. $CaPP_i$ is a competitive inhibitor of PPase, with reported K_{diss} values of ≤0.07 μM (pH 7.65, 5–8 °C; Ridlington & Butler, 1972) and 27 μM (pH 7.2, 20–22 °C; Rapoport et al., 1973). As shown in Figure 3, in the presence of a highly saturating concentration of $CaPP_i$ (1 mM), enzymatic activity is completely protected, in agreement with earlier results (Heitmann & Uhlig, 1974), and PhGx incorporation is considerably reduced. These results provide strong evidence that the unusually reactive Arg residue whose modification leads to PPase inactivation is present at the active site.

Identification of the [^{14}C]PhGx-Labeled Arginine Whose Modification Results in Loss of Enzymatic Activity. PPase contains six Arg residues per subunit, which are located at positions 5, 51, 77, 80, 182, and 189 in the covalent structure, as determined in the recent work of Cohen et al. (1978). The remainder of the Results section describes the procedures we employed to identify the highly reactive Arg residue involved in PPase activity. The overall strategy is summarized in Scheme I.

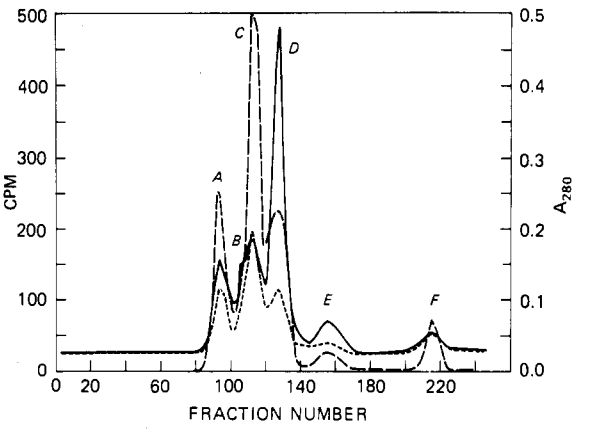


FIGURE 4: Sephadex G-50 separation of reduced and alkylated CNBr cleavage products of [^{14}C]PhGx-labeled PPase. PPase (10 mg/mL) dissolved in 70% HCO_2H was treated with a 1000-fold molar excess of CNBr, added either as a solid or in a 70% HCO_2H solution (~300 mg/mL). The reaction mixture was flushed with N_2, incubated in the dark at room temperature for 6 h, diluted with H_2O, lyophilized, and redissolved at a concentration of 10 mg/mL in 6 M urea (deionized) in 0.1 M morpholinoethanesulfonate, pH 6.35. A 500-fold molar excess of dithiothreitol was added, and the solution, reflushed with N_2, was incubated at room temperature for 20 min. Sufficient *N*-ethylmaleimide (1 M solution in propanol) was added to react with all thiol groups present, and the reaction was continued for 10 min. Following the addition of 1 mL of 20% glycerol–10% HCO_2H, the solution was applied to a Sephadex G-50 column and eluted with 10% HCO_2H at 4 °C. Column dimensions, 5 × 200 cm; fraction volume, 7.5 mL; flow rate, 30–40 mL/h. Radioactivity: no $CaPP_i$ (—); with $CaPP_i$ (---); A_{280} (– – –). Peak [identity as designated by Cohen et al. (1978)]: A (intact PPase); B (CNBr II disulfide dimer, residues 46–142); C (CNBr III, residues 143–285); D (CNBr II, residues 46–142); E (residues 46–116); F (CNBr I, residues 1–45).

Table II: Incorporation of [^{14}C]PhGx into CNBr Fragments[a]

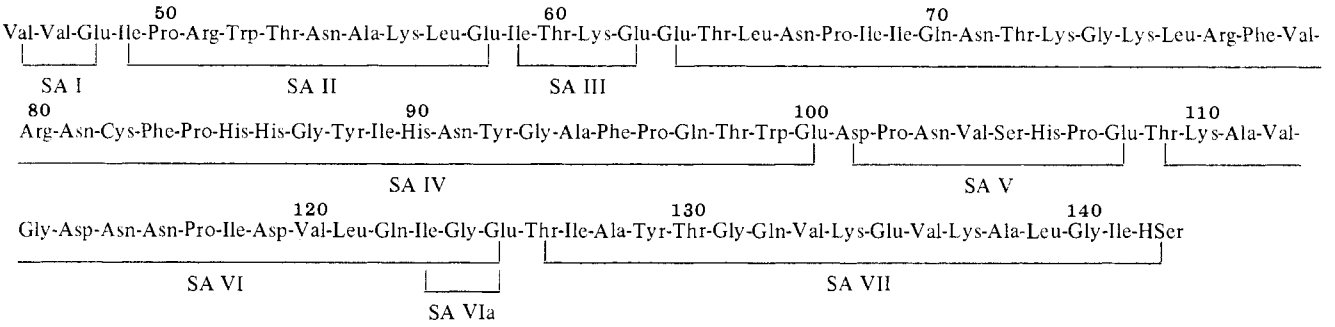
peptide	identity (residues)		cpm/nmol	mol of PhGx/mol of fragment[b]
CNBr I	1–45	$-CaPP_i$	31	0.020
		$+CaPP_i$	44	0.029
CNBr II	46–142	$-CaPP_i$	851	0.56
		$+CaPP_i$	89	0.058
CNBr III	143–285	$-CaPP_i$	315	0.206
		$+CaPP_i$	302	0.198
PPase subunit (CNBr I + CNBr II + CNBr III)		$+CaPP_i$	1197	0.78
		$-CaPP_i$	422	0.28

[a] See Figure 4. PPase was inactivated to 70% residual activity. [b] PhGx specific radioactivity is 1530 cpm/nmol. Molarity of fragments was determined by amino acid analysis and is subject to an error of ±5%.

PhGx labeling was limited to a level corresponding to 30% activity loss in order to maximize the fraction of PhGx incorporation into the Arg of interest. As indicated in Figure 2, as the level of inactivation passed 50–70%, this fraction decreased.

Steps 1, 2, and 3. [^{14}C]PhGx-Labeled PPase and Separation of CNBr Fragments. CNBr II Contains the Specifically Labeled Arginine. PPase inactivated to ~30% by incubation for an appropriate time with [^{14}C]PhGx (1.53×10^3 cpm/nmol) in the absence of $CaPP_i$ and separately PPase incubated under identical conditions but in the presence of $CaPP_i$ were first freed from excess [^{14}C]PhGx. Each sample was next treated with CNBr, and the resulting mixtures of fragments were reduced, alkylated and immediately separated on Sephadex G-50. The resulting elution patterns are shown in Figure 4. The distribution of [^{14}C]PhGx in the CNBr fragments is presented in Table II. These results show that PhGx incorporation into CNBr II is specific in that it is prevented by

Scheme II: SAV8P Digest Pattern of CNBr II[a]



peak[b]	peptide
A	SA IV and intact CNBr II
B	SA VII
C	SA VI
D	SA II
E, F	not analyzed
G	SA I (35%), SA III (33%), SA VIa (32%)

[a] Sequence taken from Cohen et al. (1978). [b] See Figure 5.

$CaPP_i$. By contrast, incorporation into either CNBr I or CNBr III is essentially unaffected by $CaPP_i$. Since PhGx incorporation in the presence of $CaPP_i$ does not effect PPase activity, PPase activity loss must be due solely to the differential PhGx incorporation into CNBr II in the absence and presence of $CaPP_i$. CNBr II must therefore contain the Arg residue of interest.

The overall labeling of PPase, as calculated by addition of the labeling of CNBr I, CNBr II, and CNBr III (Table II), is 0.78 PhGx residue/subunit of PPase in the absence of $CaPP_i$ and 0.28 residue in the presence of $CaPP_i$. The differential labeling, amounting to 0.50 PhGx residue/subunit, is a little lower than the expected value of 0.6 residue/subunit for 30% inactivated enzyme, assuming the $CaPP_i$-protected Arg is derivatized with two PhGx residues. This difference is reasonably attributable to losses of PhGx during CNBr cleavage and CNBr fragment isolation and leads to an estimated yield for PhGx recovery of 83% (0.50/0.60) for steps 1, 2, and 3. This yield must be considered a lower limit since formation of any ArgPhGx monoadduct (Werber et al., 1975) would give a lower than expected PhGx incorporation.

From the data in Table II, PhGx incorporation per PPase subunit is 2.8-fold greater in the absence than in the presence of $CaPP_i$, which agrees very well with a corresponding value of 2.7-fold for 30%-inactivated PPase, as estimated from the data in Figure 3. The overall incorporation of PhGx into 30%-inactivated PPase is estimated to be 0.78 PhGx residue/PPase subunit from the data in Table II and 0.66 and 0.60 PhGx residue/PPase subunit from the data in Figures 2 and 3, respectively. The latter two values agree within experimental error. The difference between the average of the two, 0.63 PhGx residue/PPase subunit, and the value of 0.78 PhGx residue/PPase subunit most likely arises as a result of a difference in the method of calculating PPase subunit concentration. In Table II, PPase subunit concentration depends on CNBr fragment concentration, as determined by amino acid analysis. For the experiments described in Figures 2 and 3, subunit concentrations were determined by A_{280} measurements (see Experimental Section) on solutions of redissovled, dried PPase. This determination assumes a subunit molecular weight based on the covalent structure of PPase and inherently overestimates PPase subunit concentration and thus underestimates PhGx incorporation per subunit, to the extent that dried samples of PPase used for A_{280} measurements contain tightly bound water. For this reason, we consider the higher value of PhGx incorporation to be more reliable. This higher value also conforms more closely to what would be expected if loss of PPase activity were correlated with modification of a single arginine with two PhGx residues, since the $CaPP_i$ protection experiments show that one-third of PhGx incorporation in the absence of $CaPP_i$ is unrelated to PPase inactivation.

In the experiments described in Table II and Figure 4, the identities of the peptides obtained were confirmed by amino acid composition and end group analysis and are in accord with those reported earlier by Sterner et al. (1974). Since the CNBr reaction time was shortened [6 h vs. the 48 h of Sterner et al. (1974)], less extensive nonmethionyl cleavages took place. A detectable amount of a peptide consisting of residues 46–116 was formed, probably due to acid hydrolysis of the labile Asn–Pro bond (Piszkiewiez et al., 1970). One other fragment was produced on generating (non-PhGx-labeled) CNBr fragments from low activity (320–560 IU/mg) PPase. This peptide eluted between CNBr II and residues 46–116, had Pro as the amino terminus, corresponded in amino acid composition to residues 198–285, and was never seen when high activity (560 IU/mg) material was used.

Steps 4 and 5. Proteolysis of [^{14}C]PhGx-Labeled CNBr II with S. aureus V8 Protease (SAV8P) and Separation of SAV8P Fragments. The Specifically Labeled Arginine Is either Arg-77 or Arg-80. [^{14}C]PhGx-labeled CNBr II derived from PPase labeled in the absence of $CaPP_i$ (specifically labeled CNBr II) was digested with SAV8P, and the resulting mixture of peptides was first clarified by low-speed centrifugation and then fractionated on Biogel P-4. In a parallel control experiment, CNBr II, derived from unmodified PPase, was first citraconylated and then labeled with [^{14}C]PhGx to a level of ~3 mol of PhGx per mol of CNBr II (see Experimental Section). Following Sephadex G-25 gel filtration in 10% HCO_2H, this modified CNBr II (randomly labeled CNBr II) was subjected to SAV8P proteolysis and the resulting mixture of peptides was fractionated on Biogel P-4 as above. The elution profiles are shown in Figure 5. The peptides obtained, which were characterized both by amino acid analysis and by comparison with the sequence data of Cohen et al. (1978), are identified in Scheme II. The results show that Arg-51 (peak D, SA II), which is modified in the randomly labeled sample, is not modified in the specifically labeled

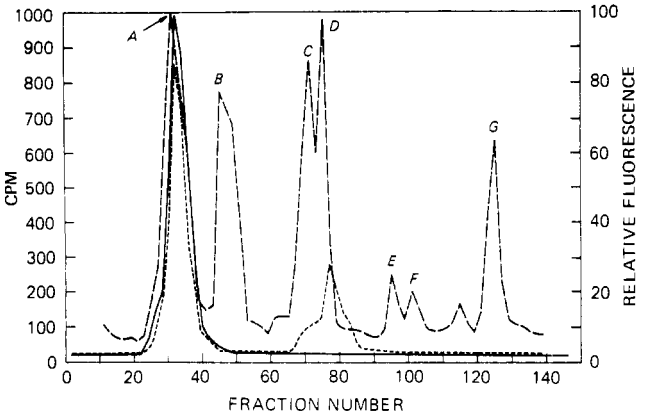


FIGURE 5: Biogel P-4 separation of peptides from SAV8P digestion of [^{14}C]PhGx-labeled CNBr II. Fragment CNBr II (100 nmol), either specifically or randomly labeled with [^{14}C]PhGx, was suspended in 1 mL of 0.1 M NH_4OAc, pH 4.0. SAV8P (Miles Research) was dissolved in the same buffer at 0.5 mg/mL, as determined spectrophotometrically assuming $A_{280}^{1\%}$ = 8.0 (Drapeau et al., 1972). Digestion was carried out at 25 °C, at a protease/substrate ratio of 1:50 by weight. Sample suspensions of specifically labeled CNBr II cleared within 2 to 3 h of the start of digestion; suspensions of randomly labeled CNBr II cleared substantially within 15 h. Additional aliquots of protease (freshly dissolved) were added at 8 and 16 h. Thawed digest mixtures were clarified by centrifugation, and the peptides in the supernatant were fractionated by gel filtration on Biogel P-4 (200–400 mesh). Column dimensions, 1.5 × 170 cm; fraction volume, 1.7 mL; flow rate, 1.3 mL/h; eluting solvent, 10% HCO_2H–10% PrOH. Radioactivity: specifically labeled peptides (—); randomly labeled peptides (---); relative fluorescence by fluorescamine analysis (see Experimental Section) (– – –).

sample. Thus, the residue of interest must be either Arg-77 or Arg-80.

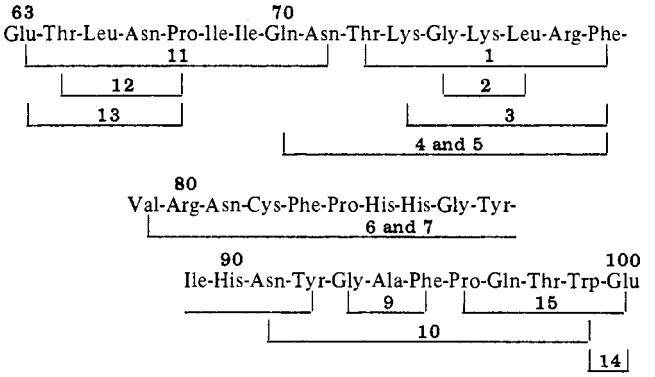
The material from peak A (Figure 4) of the specifically labeled digest mixture was rechromatographed on Sephadex G-50 (2.5 × 180 cm) in 10% HCO_2H–10% PrOH to obtain pure SA IV. This fraction contained a mixture of residues 63–100 and 64–100 (note that residues 62 and 63 are both Glu). The specific radioactivity of this peptide was 765 cpm/nmol or 90% that of the parent CNBr II fragment (Table II). This small decrease is most reasonably attributed to release of PhGx from modified peptide during SAV8P digestion, since small amounts of free PhGx were detected on the Biogel P-4 column (see below).

The amino acid analysis of SA IV derived from specifically labeled CNBr II shows a recovery of 1.4 of the 2.0 Arg residues, whereas the yields of Lys and the other amino acids are those expected from the composition of the peptide. Since Takahashi (1968) has demonstrated that PhGx-derivatized Arg is destroyed on hydrolysis, this result provides direct evidence that PhGx modification occurs at Arg rather than at Lys residues, in accord with previous work (Takahashi 1968, 1977a,b).

The peptides obtained in SAV8P digestion were those expected from the specificity of the protease with two exceptions. The protease failed to cleave at Glu-135, possibly because of the positive charge of Lys-134 (R. L. Heinrikson, personal communication). SA VIa is probably the result of deamidation of Gln-122. The amide moiety of Gln is rather labile, and its hydrolysis can occur when it is exposed to prolonged incubation in acid, although this was the only residue in which this was detected.

[^{14}C]PhGx released from the peptide during digestion was detected in fractions collected after peak G (not shown). For digests of randomly labeled CNBr II, this amounted to 62% of the total radioactivity on the column; for specifically labeled CNBr II, this amounted to 12%. For the randomly labeled

Scheme III: Peptic Digestion Pattern of SA IV[a]



[a] Sequence from Cohen et al. (1978). Peptide numbers refer to the spot compositions shown in Figure 6 and Table III.

Table III: Peptic Peptides of SA IV[a]

peptide spot no.[b]	yield (nmol)[c] randomly labeled	specifically labeled	unlabeled
1	3.0	+	+
2	0.64	+	+
3	0.43	+	+
4	0.26	0.18	+
5	0.64	0.48	–
6	2.4	5.5	+
7	0.96	–	–
8	0.51	+	+
9	5.4	+	+
10	0.58	+	+
11	0.96	+	+
12	0.83	+	+
13	3.7	+	+
14	2.0	+	+
15	1.8	+	+

[a] (+) Spot present but not analyzed; (–) not present. [b] See Figure 6. [c] Average yield (±25%) per 10-nmol digest mixture.

peptide digest, the specific radioactivity of SA II was about one-third that of the parent CNBr II fragment, after taking into account the label loss during hydrolysis.

Steps 6 and 7. Proteolysis of [^{14}C]PhGx-Labeled SA IV with Pepsin and Separation of Pepsin Fragments. The Specifically Labeled Arginine Is Arg-77. [^{14}C]PhGx-labeled SA IV derived from PPase labeled in the absence of $CaPP_i$ (specifically labeled SA IV) was digested with pepsin, and the resulting mixture of peptides was subjected to a two-dimensional thin-layer electrophoresis and chromatography fingerprinting procedure. As controls, identical pepsin digestion and fingerprinting procedures were performed on SA IV derived from unmodified PPase (unlabeled SA IV) and on SA IV derived from unmodified PPase which was labeled with [^{14}C]PhGx to a level of ~2 mol of PhGx per mol of SA IV (randomly labeled SA IV). The latter sample was prepared by using a protocol identical with that described above for the preparation of randomly labeled CNBr II.

The results of the fingerprinting procedure are shown in Figure 6. The numbered spots correspond to peptides whose compositions are shown in Scheme III. A summary of the occurrence and yields of peptides obtained from the three different samples is presented in Table III. Randomly labeled SA IV has only two peptides, spots 5 and 7, containing radioactivity, with spot 5 containing Arg-77 and spot 7 containing Arg-80. Both spots 5 and 7 are missing from the map of unlabeled SA IV, confirming their identity as peptides containing derivatized Arg. Specifically labeled SA IV con-

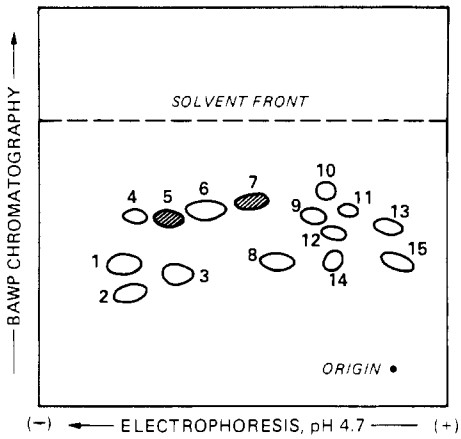


FIGURE 6: Two-dimensional analysis of peptic digest of SA IV. A portion of SA IV (10–12 nmol) was dissolved in 10 μL of 5% HCO_2H containing pepsin (0.5 mg/mL) (Sigma, 2× recrystallized from hog stomach mucosa). After incubation at 30 °C for 12 h, a second aliquot of pepsin (freshly prepared) was added and the incubation continued for a total time of 24 h. The sample was then lyophilized, redissolved in 10 μL of a buffer (1 part pyridine, 1 part glacial acetic acid, 2 parts butanol, and 36 parts water by volume, pH 4.7), and spotted on a 20 × 20 cm microcrystalline cellulose plate (Avicel 250 μm, Analtech). The plate was sprayed with the same buffer, and the sample was electrophoresed at 600 V for 75 min. After the plate was dried, chromatography was performed in the ascending mode in BAWP. Peptides were visualized as fluorescent spots after the plate was sprayed first with 10% triethylamine in acetone, then with 0.01% fluorescamine in acetone, and finally with the triethylamine solution a second time and recovered from the plate by extraction with 5 × 500 μL of 6 N HCl. Aliquots were removed for radioactivity and amino acid analysis. Peptides are identified in Scheme III. Radioactive peptides are cross-hatched.

tains spot 5, but no fluorescamine-sensitive material was found in the region of spot 7. Furthermore, when the area corresponding to spot 7 was eluted and analyzed, no peptide (<0.075 nmol) was found. Our inability to detect spot 7 in the specifically labeled sample cannot be due to a problem of poor recovery of this peptide, since on digestion and analysis of randomly labeled SA IV spot 7 is recovered in even higher yield than spot 5. From our results, Arg-80 is modified in specifically labeled SA IV to at most a very small extent, corresponding to no more than 10% of the extent of modification of Arg-77. We conclude that Arg-77 is the residue whose modification by PhGx leads to inactivation of PPase in a reaction that can be protected against by $CaPP_i$.

The sample of specifically labeled SA IV subjected to pepsin digestion was derivatized to the extent of 0.44 PhGx/peptide. Assuming a diadduct, the maximum expected yield of spot 5 from 10 nmol of such specifically labeled SA IV would be 2.2 nmol. The amount of spot 5 recovered was actually 0.48 nmol, corresponding to a 25% yield, which is typical for this system.[3] The specific radioactivity of spot 5, as recovered from the plate, was 2000 cpm/nmol, corresponding to ~1.2 PhGx/peptide. The difference of this value from 2.0 PhGx/peptide reflects loss of PhGx from the peptide on chromatography in the BAWP solvent and on elution from the plate.

It is worth noting that the major peptide containing underivatized Arg-77 (spot 1) is of different composition than spot 5. Spot 4 has the same amino acid composition as spot 5 but is produced in low yield. This may simply be a reflection of a difference in the rate of pepsin cleavage depending on whether Arg-77 is derivatized. Plates electrophoresed for times (30, 45, and 60 min) shorter than the standard time of 75 min failed to reveal any peptides with a mobility greater than that of spot 1. Also, plates run with larger (up to 25 nmol) amounts of randomly labeled digest mixture did not show any spots differing from those in Figure 5 (i.e., peptides produced in low yield which might not have been detected in the mapping of 10–12 nmol of digest).

[3] S. Franklin (personal communication).

Discussion

The use of PhGx to derivatize active-site arginine residues presents the advantages that the reagent is highly specific for arginine and can be readily obtained in radioactive form but suffers from the disadvantage that the $Arg(PhGx)_2$ adduct formed is labile at neutral pH. Furthermore, both the formation and hydrolysis of this adduct appear to be complex processes, the details of which are not fully understood. These difficulties, which are encountered not only with PhGx but also with other α-dicarbonyl reagents used in arginine modification studies as well (most notably 1,2-cyclohexanedione and 2,3-butanedione), have limited attempts to locate labeled arginine residues within enzyme covalent structures, although in the last few years labeled arginines have been located in ribonuclease A (Takahashi, 1968; Patthy & Smith, 1975b; Iijima et al., 1977), pepsin (Huang & Tang, 1972), carboxypeptidases A and B (Riordan, 1973; Werber et al., 1975), enolase (Elliot & Brewer, 1977), rhodanese (Weng et al., 1978), and adenylate kinase (Berghauser & Schirmer, 1978). For those studies which employed PhGx (Takahasi, 1968; Werber et al., 1975; Weng et al., 1978; Berghauser & Schirmer, 1978), peptides were obtained by proteolysis with trypsin and/or chymotrypsin at pH 7–8. Although such proteolysis was always accompanied by substantial losses of PhGx from modified protein, sufficient PhGx was retained to allow identification of derivatized peptides. We originally planned to follow a similar procedure in our own mapping studies. However, several observations made in the course of this work indicated that, at least in our case, such a procedure could yield ambiguous results. First, the CNBr cleavage results (Table II) showed that more than one Arg was being substantially labeled. Second, incubation of specifically labeled PPase at pH 8 for 1 h at 37 °C and for 45 min at room temperature led to loss of 63% of the covalently incorporated PhGx. Third, we found that rates of loss of PhGx from NAc-$Arg(PhGx)_2$ and from specifically labeled PPase and randomly labeled PPase and PPase fragments differed substantially from one another under identical incubation conditions.[4] Fourth, our study of $NAcArg(PhGx)_2$ hydrolysis at pH 8 implies that at this pH PhGx can come off and subsequently recondense with Arg residues. Although in our model study the second phase of reaction, presumably corresponding to reuptake of

[4] The underlying reasons for these differences in stability have not been established. One possibility is that the local environment plays a major role in determining stability. Another is that more than one isomer of $NAcArg(PhGx)_2$ may be formed, and different isomers may have different stabilities. Two possible structures have been proposed for $NAcArg(PhGx)_2$ (Takahashi, 1968), and it is not presently known which of the proposed structures is correct or if $NAcArg(PhGx)_2$ is a mixture of the two. Moreover, each structure contains three chiral carbons in addition to the α carbon of Arg, raising the possibility of the formation of multiple diastereomeric forms, each with its own stability toward PhGx loss. In this connection we have already obtained evidence for at least some heterogeneity in the structure of $NAcArg(PhGx)_2$ from its ^1H NMR spectrum (not shown), in which the two originally aldehydic protons in the two PhGx residues show up as three singlet peaks at δ values from tetramethylsilane (external) of 5.62, 5.38, and 5.24 ppm (solvent methanol-d_4) and integrate for 1.0, 0.7, and 0.3 proton, respectively. Considerable heterogeneity has also been found for 1,2-cyclohexanedione (Patthy & Smith, 1975a) and kethoxal (Iijima et al., 1977) adducts to arginine.

PhGx, is quite slow, in the presence of a high local Arg concentration, such as might exist on the surface of a protein, this second phase might proceed much more rapidly. Taken together, these observations raised the possibility that in a mapping procedure involving large losses of PhGx, the observed distribution of PhGx label over the various Arg residues might not reflect the distribution when native protein is being labeled.

Accordingly, we sought to develop a new mapping procedure which would minimize PhGx loss. The results of our studies on the hydrolysis rate of $NAcArg(PhGx)_2$ indicated the desirability of a procedure utilizing low pH, which is provided by the combination of the CNBr cleavage and SAV8P and pepsin proteolysis steps. We estimate the yield of PhGx recovery in steps 1, 2, and 3 to be at least 83% (see Results). At least part of the PhGx loss in this step is attributable to the reduction/alkylation step, which requires a 0.5-h incubation at room temperature at pH 6.35. The lack of migration of PhGx between Arg residues during isolation of CNBr II is evidenced by the large difference in distribution of label in the experiments performed in the absence and presence of $CaPP_i$ (Table II). The yield of PhGx recovery in labeled SA IV derived from labeled CNBr II is ~90%, as calculated from the specific radioactivity of SA IV compared to that of CNBr II. In many cases, these two steps would be sufficient for identification of a labeled Arg residue. In the present example, the proximity of Arg-80 to Arg-77 made necessary the additional step of pepsin digestion.

Our results provide conclusive evidence for the identity of Arg-77 as the site of specific (i.e., $CaPP_i$-protected) PhGx modification. This residue falls within spot 5, the only radioactive peptide obtained on pepsin digestion of specifically labeled SA IV (Scheme III). The only other plausible sites for PhGx modification within spot 5 are Lys-73 and Lys-75, and there are three lines of evidence which taken together clearly show that neither of these residues is derivatized. First, spot 5 was also obtained on pepsin digestion of randomly labeled SA IV, where there is no possibility of Lys labeling because of the use of the citraconylation procedure (see Experimental Section). Second, the work of Takahashi (1968, 1977a,b) indicates a very marked selectivity of PhGx for Arg as opposed to Lys side chains. Our earlier work (Cooperman & Chiu, 1973b), confirmed in the present studies (data not shown), showed that PhGx inactivation of PPase proceeded with a second-order rate constant 4–5 times that expected for an isolated Arg, and thus very much faster than what would be expected for reaction with a Lys residue. Third, the amino acid analysis of specifically labeled SA IV shows less than stoichiometric amounts of Arg, as would be expected for Arg derivatization (Takahashi, 1968), but the full stoichiometry of Lys.

Is Arg-77 an essential residue, in the sense that it is directly involved in the catalytic mechanism? Our results, showing that PhGx modification of a single Arg leads to essentially full inactivation, that inactivation and Arg-77 modification are blocked by the competitive inhibitor $CaPP_i$, and earlier (Cooperman & Chiu, 1973b) that PhGx modification results in a loss of competitive inhibitor binding, are certainly consistent with this interpretation. However, as with any such chemical modification study, it cannot be ruled out that Arg-77 is just near the active site rather than essential or even that the ability of $CaPP_i$ to protect against Arg-77 modification and enzyme inactivation reflects an allosteric phenomenon rather than a direct local site effect.

PPase displays a pH optimum between 6.5 and 7.0 (Kunitz, 1952; Konsowitz & Cooperman, 1976). At this pH, essentially all Lys and Arg residues should be positively charged and some His residues as well. Such residues would be expected to be important for binding the pyrophosphate multianion to the enzyme. It is thus worth noting that Arg-77 falls within an octadecapeptide (residues 73–90) of especially high positive charge density, containing 2 Lys, 2 Arg, and 3 His residues out of a total of 28 Lys, 6 Arg, and 6 His per subunit (Cohen et al., 1978). In addition, the low level of derivatization of Arg-5, Arg-51, and Arg-80 on treatment of PPase with PhGx is an indication that these residues may be inaccessible to solvent in the native structure.

As mentioned in the portion of the Results section describing steps 1, 2, and 3, we found that Sephadex G-50 chromatography of the CNBr cleavage products of lower specific activity PPase led to isolation of a fragment, residues 198–285. This fragment might arise from hydrolysis of PPase by a contaminating protease (Eifler et al., 1972). If so, it could account for the report by Avaeva et al. (1968, 1972) that PPase has an $\alpha_2\beta_2$ subunit structure, which is in disagreement with the findings of several other groups that PPase is composed of two identical polypeptide chains (Ridlington et al., 1972; Hansen et al., 1972; Heinrikson et al., 1973). Sklyankina et al. (1973) have also claimed that trinitrobenzenesulfonate treatment of PPase leads to full inactivation of PPase, and this disagrees with results presented by ourselves (Cooperman & Chiu, 1973b) and others (Heitmann & Uhlig, 1974). Although we cannot be sure of the reason for the disagreement, we note that a partially proteolyzed PPase would contain an α-amino group not present in intact PPase, and derivatization of such a group could lead to inactivation.

Acknowledgments

We acknowledge the assistance in various portions of this work of Drs. R. A. Hogue-Angeletti (University of Pennsylvania, amino acid analysis), M. Weigert and S. G. Franklin (Institute for Cancer Research, Philadelphia, two-dimensional peptide analysis), and R. L. Heinrikson and R. Sterner (University of Chicago). Sam Zaremba and Paul Russ helped with the kinetics of hydrolysis of N^{α}-acetyl(diphenylglyoxal)arginine.

References

Anderson, C. M., Stenkamp, R. E., McDonald, R. C., & Steitz, T. A. (1978) *J. Mol. Biol. 123*, 207.

Avaeva, S. M., Braga, E. A., & Egorov, A. M. (1968) *Biofizika 13*, 1126.

Avaeva, S. M., Lebedeva, Z. I., Beisembaeva, R. U., & Egorov, A. M. (1972) *FEBS Lett. 24*, 169.

Berghauser, J., & Schirmer, R. H. (1978) *Biochim. Biophys. Acta 537*, 428.

Blow, D. M. (1976) *Acc. Chem. Res. 9*, 145.

Brunswick, D. J., & Cooperman, B. S. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1801.

Bunick, G., McKenna, G. P., Scarbrough, F. E., Uberbacher, E. C., & Voet, D. (1978) *Acta Crystallogr., Sect. B 34*, 3210.

Cohen, S. A., Sterner, R., Keim, P. S., & Heinrikson, R. L. (1978) *J. Biol. Chem. 253*, 889.

Cooperman, B. S., & Chiu, N. Y. (1973a) *Biochemistry 12*, 1670.

Cooperman, B. S., & Chiu, N. Y. (1973b) *Biochemistry 12*, 1676.

Cooperman, B. S., Chiu, N. Y., Bruckmann, R. H., Bunick, G. J., & McKenna, G. P. (1973) *Biochemistry 12*, 1665.

Cotton, F. A., Day V. W., Hazen, E. E., Jr., Larsen, S., & Wong, S. T. K. (1974) *J. Am. Chem. Soc. 96*, 4471.

Dixon, H. B. F., & Perham, R. N. (1968) *Biochem. J. 109*, 312.
Drapeau, G. R., Boily, Y., & Houmard, J. (1972) *J. Biol. Chem. 247*, 6720.
Eifler, R., Hahn, V., & Hermann, I. (1972) *Acta Biol. Med. Ger. 28*, 707.
Elliot, J. I., & Brewer, J. M. (1977) *Arch. Biochem. Biophys. 190*, 351.
Gray, W. R. (1972) *Methods Enzymol. 25*, 121.
Habeeb, A. F. S. A., & Atassi, M. Z. (1970) *Biochemistry 9*, 4939.
Hackney, D., & Boyer, P. D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3133.
Hamm, D. J., & Cooperman, B. S. (1978) *Biochemistry 17*, 4030.
Hansen, G., Eifler, R., & Heitmann, P. (1972) *Acta Biol. Med. Ger. 28*, 977.
Heinrikson, R. L., Sterner, R., Noyes, C., Cooperman, B. S., & Bruckmann, R. H. (1973) *J. Biol. Chem. 248*, 2521.
Heitmann, P., & Uhlig, H. J. (1974) *Acta Biol. Med. Ger. 32*, 565.
Huang, W.-Y., & Tang, J. (1972) *J. Biol. Chem. 247*, 2704.
Iijima, H., Patrzyc, H., & Bello, J. (1977) *Biochim. Biophys. Acta 491*, 305.
Konsowitz, L. M., & Cooperman, B. S. (1976) *J. Am. Chem. Soc. 98*, 1993.
Kraut, J. (1977) *Annu. Rev. Biochem. 46*, 331.
Kunitz, J. (1952) *J. Gen. Physiol. 35*, 423.
Levine, M., Muirhead, H., Stammers, D. K., & Stuart, D. I. (1978) *Nature (London) 271*, 626.
Makhaldiani, V. V., Smirnova, E. A., Voronova, A. A., Kuranova, I. P., Arutyunyun, E. G., Vainshtein, B. K., Hoehne, W. F., Binwald, B., & Hansen, G. (1978) *Dokl. Akad. Nauk SSSR 240*, 1478.
Moe, O. A., & Butler, L. G. (1972) *J. Biol. Chem. 247*, 7308.
Pai, E. F., Sachsenheimer, W., Schirmer, R. H., & Schulz, G. E. (1977) *J. Mol. Biol. 114*, 37.
Patthy, L., & Smith, E. L. (1975a) *J. Biol. Chem. 250*, 557.
Patthy, L., & Smith, E. L. (1975b) *J. Biol. Chem. 250*, 565.
Piszkiewiez, D., Landon, M., & Smith, E. L. (1970) *Biochem. Biophys. Res. Commun. 40*, 1173.
Rapoport, T. A., Hohne, W. E., Heitmann, P., & Rapoport, S. (1972) *Eur. J. Biochem. 26*, 237.
Rapoport, T. A., Hohne, W. E., Heitmann, P., & Rapoport, S. (1973) *Eur. J. Biochem. 33*, 341.
Ridlington, J. W., & Butler, L. G. (1972) *J. Biol. Chem. 247*, 7303.
Ridlington, J. W., Yang, Y., & Butler, L. G. (1972) *Arch. Biochem. Biophys. 153*, 714.
Riley, H. A., & Gray, A. R. (1947) *Organic Syntheses*, Collect. Vol. II, p 509, Wiley, New York.
Riordan, J. F. (1973) *Biochemistry 12*, 3915.
Riordan, J. F., McElvany, K. D., & Borders, C. L., Jr. (1977) *Science 195*, 884.
Sklyankina, V. A., Medvedva, I. V., & Avaeva, S. M. (1973) *Dokl. Akad. Nauk SSSR, Ser. Biol. 211*, 494.
Sterner, R., Noyes, C., & Heinrikson, R. L. (1974) *Biochemistry 13*, 91.
Takahashi, K. (1968) *J. Biol. Chem. 243*, 6171.
Takahashi, K. (1977a) *J. Biochem. (Tokyo) 81*, 395.
Takahashi, K. (1977b) *J. Biochem. (Tokyo) 81*, 403.
Udenfreund, S., Stein, S., Bohlen, P., Dairman, W., Leimgruber, W., & Weigle, M. (1972) *Science 178*, 871.
Weiner, A. M., Platt, T., & Weber, K. (1972) *J. Biol. Chem. 247*, 3242.
Weng, L., Heinrikson, R. L., & Westley, J. (1978) *J. Biol. Chem. 253*, 8109.
Werber, M. M., Moldovan, M., & Sokolovsky, M. (1975) *Eur. J. Biochem. 53*, 207.

Human Erythrocytic Purine Nucleoside Phosphorylase: Reaction with Sugar-Modified Nucleoside Substrates†

Johanna D. Stoeckler, Carolyn Cambor, and Robert E. Parks, Jr.*

ABSTRACT: The kinetic parameters (K_m and V_{max}) of sugar-modified analogues of inosine and guanosine have been determined with human erythrocytic purine nucleoside phosphorylase (PNP). Steric alterations at the 2′ and 3′ positions greatly lessened or abolished substrate activity. However, the 5′-deoxy- and 2′,5′-dideoxy-β-D-ribofuranosyl and the α-L-lyxosyl analogues were good substrates, indicating that the 5′-hydroxyl and the orientation of the 5′-hydroxymethyl group are not important for binding. The sugar phosphate analogue, 5-deoxyribose 1-phosphate, was synthesized from 5′-deoxyinosine with immobilized PNP, and its presence was verified by using it in the enzymic synthesis of 5′-deoxyguanosine. The adenosine versions of the 5′-modified analogues were also found to react with adenosine deaminase, albeit at <1% of V_{max}.

Human erythrocytic purine nucleoside phosphorylase (purine nucleoside:orthophosphate ribosyltransferase, PNP, EC 2.4.2.1) has been purified to homogeneity, and its physical properties and enzymatic behavior have been studied extensively (Krenitsky et al., 1968; Parks & Agarwal, 1972; Turner et al., 1971; Agarwal, K. C., et al., 1975; Agarwal et al., 1978; Stoeckler et al., 1978a,b; Zannis et al., 1978). This enzyme catalyzes the reversible phosphorolysis of ribonucleoside and 2′-deoxyribonucleoside derivatives of hypoxanthine, guanine, xanthine, and many of their analogues by the reaction

purine nucleoside + P_i ⇌ purine base + pentose 1-phosphate

Currently there is great interest in PNP for several reasons. A genetic disease has been identified that involves a specific

† From the Section of Biochemical Pharmacology, Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912. *Received August 23, 1979.* This work was supported by a grant from the American Cancer Society (ACS CH-7S and CH-7T) and a U.S. Public Health Service grant (CA 13943).

0006-2960/80/0419-0102$01.00/0