Mechanism of Allosteric Activation of Glycogen Phosphorylase Probed by the Reactivity of Essential Arginine Residues. Identification of an Arginine Residue Involved in the Binding of Glucose 1-Phosphate†

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ABSTRACT: We have previously reported the physicochemical and kinetic properties of glycogen phosphorylase modified by arginine-specific reagents under different conditions [Dreyfus, M., Vandenbunder, B., & Buc, H. (1980) *Biochemistry 19*, 3634–3642]. The properties of the modified enzyme depend upon the conformation adopted by the enzyme during the modification reaction. In this paper, we report the localization of the crucial modified arginine residues on the primary structure. The chymotryptic peptide extending from residue Asp-563 to residue Tyr-572 was shown to contain one arginine residue (Arg-568) which is chemically modified by phenylglyoxal in phosphorylase *a* and in activated phosphorylase *b*. Inclusion of glucose 1-phosphate in the modification medium protects this residue from modification, with a concomitant protection of the enzyme activity. Furthermore, this residue is not reactive toward phenylglyoxal in phosphorylase *b* in the absence of any effector. Addition of the AMP analogue 2′dAMP, which is not an activator of the enzyme, does not increase Arg-568 reactivity but protects from modification several arginine residues located between Arg-242 and Leu-348. The location and the role of Arg-568 in phosphorylase are discussed with reference to recent data from X-ray crystallography.

In view of increasing evidence that enzymes employ arginine residues to interact with negatively charged phosphate groups (Riordan et al., 1977), it was reasonable to look for arginine residues at the nucleotide and the glucose 1-phosphate binding sites of glycogen phosphorylase (EC 2.4.1.1). The first paper of this series (Dreyfus et al., 1980) dealt with enzymatic properties of phosphorylase modified by 2,3-butanedione, an arginine-specific protein modifying regeant. We have shown that the result of the modification depends upon the conformation of phosphorylase during the reaction. Thus, phosphorylase *b*, incubated with butanedione in the absence of any activator, loses its ability to bind adenosine 5′-monophosphate (5′AMP),[1] but not its catalytic activity. In contrast, when phosphorylase *a*, or phosphorylase *b* in the presence of AMP, is modified, rapid loss of catalytic activity occurs. A correlation exists between the rate of inactivation of phosphorylase in various conformations and the K_m of the enzyme for glucose 1-phosphate under the same conditions. Modified phosphorylase *a* no longer binds glucose cyclic 1,2-phosphate, a substrate analogue. Moreover, the addition of Glc-1-P to the modification medium causes a decrease in the inactivation rate. These results support the hypothesis that Glc-1-P protects one (or one group of) arginine residue(s) which is (are) essential for catalytic activity and which is (are) accessible to modification only in active conformations of phosphorylase. However, no evidence was provided showing that Glc-1-P interacts *directly* with this residue, for example, by making an ionic contact with it. We could not exclude the possibility of a long-distance interaction between Glc-1-P and a remote arginine residue, which would be mediated by a conformational change. Furthermore, we do not know whether the arginine residues protected against modification by the substrate Glc-1-P are the same in both phosphorylase *a* and activated phosphorylase *b*. Evidence that this residue is unique and is actually located at the substrate binding site can only come from its identification on the primary sequence and subsequently from its localization on the tertiary structure. Recent crystallographic work has provided a description of the active site of phosphorylase at high resolution (Johnson et al., 1980; Sprang & Fletterick, 1979). The possibility of referring to these three-dimensional structures prompted us to undertake the identification of the essential arginine residue(s).

In the present work, we have modified phosphorylase with phenylglyoxal, an arginine-directed reagent (Takahashi, 1968) which is available as a radioactive compound. Modification in the presence or absence of the substrate glucose 1-phosphate was performed on the "activated" conformation of the enzyme; by this we mean that, in the presence of its substrates, phosphorylase behaves like a Michaelian enzyme with high V_{max} and low K_m for substrates. Such a situation is found in phosphorylase *a* in the presence of AMP or in phosphorylase *b* in the presence of both AMP and alcohol (Dreyfus et al., 1978, 1980). As a first step in the precise localization of modified arginine residues on the amino acid sequence, we cleaved the modified enzyme with cyanogen bromide. The resulting peptides were fractionated by gel filtration, and the distribution of modified arginine residues over the elution profile was determined. Using this technique, we located the cyanogen bromide fragment whose phenylglyoxal content was reduced when Glc-1-P was added to the reaction medium. This fragment was further digested with chymotrypsin, and one unique chymotryptic peptide was identified which was modified in activated phosphorylase and which was not

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[1] Abbreviations used: 5′AMP, adenosine 5′-monophosphate; Glc-1-P, α-D-glucopyranose 1-phosphate; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; CNBr, cyanogen bromide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); $NaDodSO_4$, sodium dodecyl sulfate.

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modified when Glc-1-P was present.

Materials and Methods

Glycogen phosphorylases *b* and *a* were prepared from rabbit skeletal muscle and assayed as described in the first paper of this series (Dreyfus et al., 1980). α-Chymotrypsin was purchased from Worthington. [^{14}C]Phenylglyoxal (28 mCi/mmol) was obtained from the Commissariat à l'Energie Atomique (C.E.A., Saclay). Phenylglyoxal (Fluka A.G.) was recrystallized before use. Fresh aqueous stock solutions of phenylglyoxal were prepared daily; concentrations were determined from the absorbance of an aliquot diluted in methanol, using ϵ_{247nm} = 11 300 M^{-1} cm^{-1} (Kohlbrenner & Cross, 1978). Other chemicals used in this study were analytical grade products.

Preparation of Phosphorylase Labeled at the Catalytic Site. Phenylglyoxal modification of the "activated" conformations of phosphorylase was performed at 30 °C either on phosphorylase *a* in the presence of 0.5 mM 5'AMP or on phosphorylase *b* in the presence of 12% *tert*-butyl alcohol and 1 mM 5'AMP (Dreyfus et al., 1978). The protection offered by the substrate glucose 1-phosphate was studied by adding 5 mM Glc-1-P in the case of phosphorylase *a* and 10 mM Glc-1-P for phosphorylase *b* to the reaction medium.

Unless otherwise stated, phosphorylase (monomer concentration 50 μM) in 20 mM sodium tetraborate buffer, 1 mM EDTA, and 1 mM DTE, pH 7.5, was incubated, in the presence of appropriate effectors and ligands, with 370 μM radioactive phenylglyoxal at 30 °C. At various times, aliquots were removed and assayed for enzyme activity (Dreyfus et al., 1978) or for the incorporation of radioactivity by using a rapid filter paper assay (Reimann et al., 1971). The reaction was allowed to stand for 3–4 h, or until about 1–2 mol of phenylglyoxal was incorporated per phosphorylase subunit. The reaction was quenched by addition of an equal volume of borate buffer, followed by precipitation of the enzyme with ammonium sulfate (50% saturation) at 4 °C. After centrifugation, the pellet was suspended in 20 mM sodium tetraborate buffer containing 1 mM EDTA, pH 7.5 (final concentration 10–20 mg/mL), and dialyzed overnight against this buffer. The concentration of the labeled enzyme was measured from the absorbance at 280 nm, and aliquots were assayed for radioactivity. These measurements, run in triplicate, allowed precise determination of the molar incorporation of phenylglyoxal. In all cases, this incorporation agreed within 15% with values obtained at the end of the reaction with the filter assay system, thus ruling out extensive loss of label during dialysis.

Carboxymethylation was performed at pH 8.2 according to Saari & Fischer (1973), except that guanidine hydrochloride (6 M) was used instead of urea. The time course of the alkylation reaction was followed by DTNB titration in 0.1 M ammonium bicarbonate also containing 6 M guanidine hydrochloride. After complete alkylation, the solution was dialyzed extensively against 9% formic acid and lyophilized. Despite the reported reversibility of phenylglyoxal modification at basic pH (Takahashi, 1968) and the fact that alkylation was run in the presence of large amounts of guanidine ions, an arginine analogue, less than 10% of the protein-bound phenylglyoxal was lost during this step.

Cleavage of Labeled Enzyme. CNBr cleavage of labeled phosphorylase, gel filtration of CNBr peptides, and purification of labeled CB15 and CB17 were carried out according to Koide et al. (1978). Digestion by α-chymotrypsin was achieved at high protease-to-substrate concentration ratios (10–15% w/w) to avoid long exposure of the labeled peptides to basic pH (Takahashi, 1968). Labeled peptides (0.5–2 mg/mL) were dissolved in dilute acetic acid, and the pH was then increased to 7.6–7.8 with concentrated ammonia. Chymotrypsin was added, and the peptide suspension was stirred at 37 °C for 1 h. A second addition of chymotrypsin (half the first) was then made, and incubation was continued for 30 min. The digestion is then stopped by adding 1% formic acid. The small amount of insoluble material present at the end of the digestion contained no radioactivity.

Peptide Mapping. Peptide maps were performed on 20 × 20 plates covered with a thin layer of microcrystalline cellulose (CEL 400-10, from Macherey-Nagel, Düren, Germany, or DS-O, from Camag, Berlin, Germany) according to Yagushi et al. (1975). The electrophoresis in the first dimension was carried out for 90 min (pyridine/acetic acid/acetone/water (2:4:15:79 v/v) at 400 V. After drying, the plate was developed in the second dimension in pyridine/1-butanol/acetic acid/water (50:75:15:60 v/v). Peptide maps were sprayed either with 5 ml of a ninhydrin solution (750 mg of ninhydrin, 300 mL of methanol, 100 mL of acetic acid, and 40 mL of *sym*-collidine) or with a solution of 0.005% fluorescamine in acetone (Vandekerkhove & Van Montagu, 1974). Radioactive peptides were localized by autoradiography with NS2T films (Kodak) and eluted from the cellulose either with 10% formic acid or 50% acetic acid.

Amino Acid and Sequence Analysis. Samples were hydrolyzed for 24 h in 6 M HCl in evacuated, sealed ampules at 110 °C. Hydrolysis of phenylglyoxal-labeled peptide was carried out in the presence of 0.5% thioglycolic acid (Patthy & Smith, 1975). The amino acid analyses were performed in a Labotron Liquimat II machine. *o*-Phthalaldehyde was used in the detection system according to recommendations of Benson & Hare (1975). The purified peptide corresponding to residues 563–572 was sequenced, on a 3-nmol scale, with the dansyl–Edman method (Gray, 1972) miniaturized as recommended by Chen (1976), with the following modifications: (1) the coupling step was done in 60%, rather than 50%, aqueous pyridine, to achieve complete solubilization of the phenyl isothiocyanate, and (2) the acid cleavage step was shortened to 10 min during the second degradation cycle since a N-terminal glutamine was suspected.

Results

Identification of the Arginine Residue Protected by Glc-1-P on Phosphorylase a. Rabbit muscle glycogen phosphorylase *a* is rapidly inactivated by an excess of phenylglyoxal. Preliminary studies showed that the catalytic activity of phosphorylase *a* (50 μM) decreases exponentially to zero upon incubation with phenylglyoxal (7.5 mM) and that 50% of the activity was lost within 5 min after addition of the reagent. We have estimated the extent of arginine modification by the incorporation of [^{14}C]phenylglyoxal. Five moles of phenylglyoxal per subunit was incorporated when the enzymatic activity was reduced by 95%, and incorporation continued almost linearly with time well after all activity had been lost. These results suggested that the arginine residues which are essential for catalytic activity have a higher reactivity than the bulk of the reactive arginines and prompted us to use lower phenylglyoxal concentrations to improve the specificity of the modification reaction.

Phosphorylase *a* (50 μM) was incubated with 0.5 mM 5'AMP either in the presence of in the absence of Glc-1-P (5 mM). Modification was started by addition of [^{14}C]phenylglyoxal (370 μM), and the reaction was run for the same time in both cases, until about 1 mol of phenylglyoxal was incorporated per subunit. When glucose 1-phosphate was present in the reaction mixture, the initial rate of [^{14}C]phenylglyoxal

Table I: Reaction of Phosphorylase with Phenylglyoxal: Time Course of the Inactivation and Reagent Incorporation under Different Conditions[a]

enzyme labeled	effectors and ligands added to the modification medium	reaction time (min)	moles of phenylglyoxal incorporated/mole of phosphorylase subunit	residual activity (%)
phosphorylase *a*	0.5 mM 5'AMP	126	1.28	50 (a)
	0.5 mM 5'AMP + 5 mM Glc-1-P	126	1.11	69 (a)
phosphorylase *b*	12% *tert*-butyl alcohol + 1 mM 5'AMP	160	0.85	73 (b)
	12% *tert*-butyl alcohol + 1 mM 5'AMP + 10 mM Glc-1-P	160	0.69	87.5 (b)
phosphorylase *b*	none	255	1.65	56 (a)
	+ 10 mM 2'dAMP	255	1.35	80 (a)

[a] The enzyme, 50 μM, stabilized in the appropriate conformation, is incubated in the presence of 370 μM radioactive phenylglyoxal in borate buffer at 30 °C. After reaction, excess of reagents and effectors are removed as under Material and Methods. Reagent incorporation is measured, and the labeled enzyme is assayed either in the presence of 5'AMP (a) or in the presence of *tert*-butyl alcohol (b), as under Material and Methods.

incorporation was reduced by 19 ± 2%, corresponding to a reduction of the inactivation rate by a factor of 2.1 ± 0.2 (average of three determinations; cf. Table I). After modification, excess reagent, nucleotide, and other effectors were removed. The modified enzyme was carboxymethylated and cleaved with cyanogen bromide (Koide et al., 1978). The CNBr peptides were fractioned on a column of Sephadex G-50 superfine (Figure 1A). All fractions were radioactive, further showing that many arginines are labeled by phenylglyoxal. The radioactive profiles obtained from phosphorylase *a* labeled in the presence or absence of Glc-1-P were essentially identical, except that the radioactivity on the first peptide peak which, in the absence of Glc-1-P amounted to 35% of the total radioactivity, was reduced by 40% when modification was done in the presence of Glc-1-P (Figure 1A). Material corresponding to this peak was rechromatographed on the same column and found homogeneous by gel electrophoresis in the presence of $NaDodSO_4$ and urea (Epstein & Wolff, 1976). The amino acid composition (Table II), the presence of a single alanine residue at the N terminus, and the molecular weight of this fragment identify it as peptide CB15, described by Saari & Fischer (1973). We conclude that the arginine residue(s) protected by Glc-1-P is located in CB15, a large peptide extending from Ala-441 to Met-603 and containing 11 arginines. Fragments CB15 obtained from enzyme labeled in the presence or absence of Glc-1-P will be subsequently referred to as CB15(+) and CB15(−), respectively.

Approximately 2.5 μmol of CB15(−) and of CB15(+) was digested with chymotrypsin. Fractionation of the CB15(−) chymotryptic digest on a Bio-Gel P4 column yielded a major radioactive peak (Figure 2) which contained about 30% of the total amount of [^{14}C]phenylglyoxal incorporated in CB15(−) and which was not observed with a chymotryptic digest of CB15(+). Material from this fraction was further chromatographed on an AG 50-X8 column. Numerous nonradioactive peptides were eluted from this column before all the radioactivity emerged as a single peak. This radioactive material was applied to TLC plates and fingerprinted as described under Materials and Methods. After autoradiography, only one radioactive spot was observed; this corresponds to the most intense spot on the fluorescamine-stained map. Material eluted from this spot proved to be homogeneous. Its amino acid composition (Table II) and its N-terminal sequence (Figure 3) identify it as peptide 563–572 on the sequence of phosphorylase. Comparisons of amino acid analysis with radioactivity measurements showed that the purified material contains precisely (within ±10%) 1 mol of phenylglyoxal per mol of peptide. During sequential Edman degradation, little radioactivity was eluted from the peptide for the first five cycles, but about 60% of the theoretical radioactivity was recovered in the organic phase during the sixth cycle, corresponding to Arg-568 (Figure 3). It was found possible to extend the sequence beyond this step, thus indicating that the burst of radioactivity was not due to a washing out of the peptide but rather to a release of a radioactive amino acid at this particular cycle. The recovery of radioactivity was considered satisfactory, in view of the nonquantitative yield of the manual degradation, especially since a N-terminal glutamine was present during the second cycle.

Table II: Amino Acid Composition of Peptides Containing Crucial Arginine Residues[a]

	CB15[b]	peptide (563–572) after fingerprint[c]	CB17[b]
Cys	2.9 (3)	0 (0)	1.0 (1)
Asx	16.0 (17)	1.09 (1)	18.4 (18)
Thr	4.5 (5)		3.0 (3)
Ser	4.1 (5)		4.2 (5)
Glx	16.5 (18)	2.32 (2)	9.2 (9)
Pro	5.5 (6)		5.7 (6)
Glx	6.1 (5)	0.36 (0)	4.5 (4)
Ala	10.0 (10)		9.0 (9)
Val	11.1 (13)	2.00 (2)	6.9 (7)
Ile	11.3 (14)	0.65 (1)	6.1 (5)
Leu	16.6 (17)		11.1 (11)
Tyr	5.8 (7)	0.93 (1)	3.4 (3)
Phe	6.5 (7)		9.0 (9)
His	7.2 (7)	1.13 (1)	1.3 (1)
Lys	13.8 (16)	0.88 (1)	6.8 (7)
Arg	10.0 (11)	0.51 (0–1)	7.7 (8)

[a] Residues/molecule from amino acid analysis or (in parentheses) from the sequence (Titani et al., 1977). Amino acid analysis has been carried out respectively with 5 nmol[b] or 1.0 nmol[c] of material after acid hydrolysis at 110 °C in 6 N HCl for 24 h.

We conclude that the major peak observed on the Bio-Gel P4 elution pattern of CB15(−)-chymotryptic digest (Figure 2) corresponds to one modified arginine only, Arg-568. This residue is not modified on CB15(+). Analysis of Figures 1A and 2 shows that the protection of Arg-568 by Glc-1-P accounts for 80% of the differential [^{14}C]phenylglyoxal incorporation in phosphorylase *a* which occurs upon addition of Glc-1-P to the reaction medium.

Reactivity of Arginine-568 in Different Phosphorylase Conformations. Arg-568 has been identified on phosphorylase *a* as a residue which reacts with phenylglyoxal and is protected by Glc-1-P against modification, with simultaneous protection of activity. In a previous paper (Dreyfus et al., 1980), we showed that butanedione treatment also inactivates phosphorylases *a* and *b* through the modification of one (or one group of) arginine residue(s) which could be protected by Glc-1-P. Moreover, the rate of the inactivation process was shown to increase in the presence of allosteric activators and to decrease in the presence of allosteric inhibitors. Hence, the following questions arise: (1) Is butanedione inactivation also

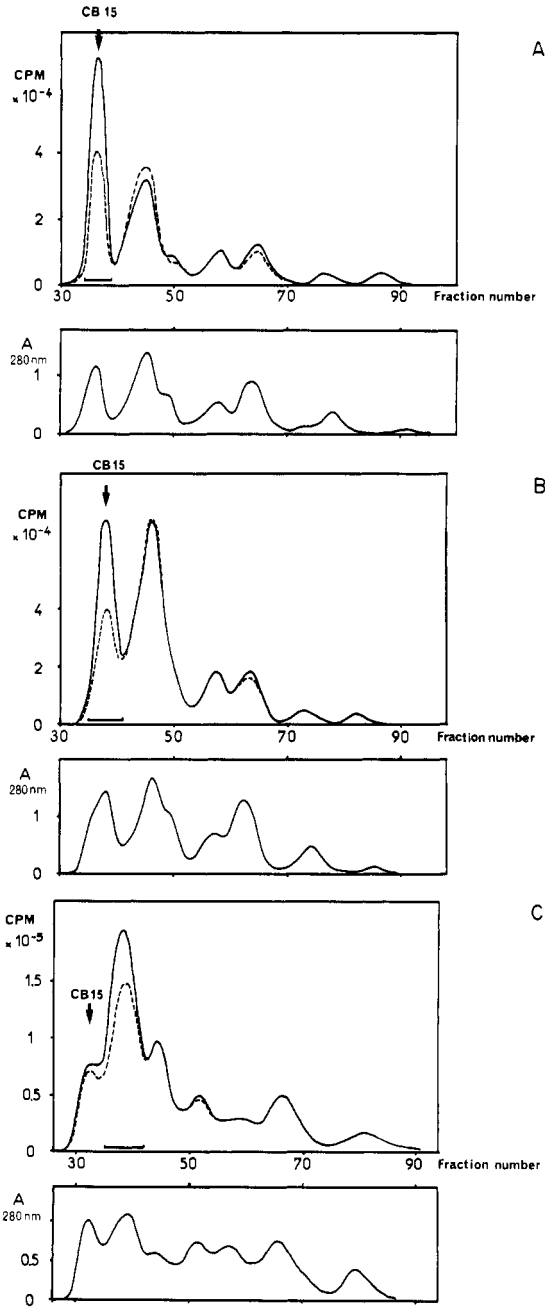

FIGURE 1: Gel-filtration pattern of CNBr peptides obtained from phosphorylase after labeling with [^{14}C]phenylglyoxal. (A) (Upper frame) CNBr fragments generated from phosphorylase *a* (~20 mg) labeled in the presence of 0.5 mM AMP and either in the absence (solid line) or in the presence (broken line) of 5 mM Glc-1-P as described under Material and Methods. The column (Sephadex G-50 superfine, 1 × 80 cm) was eluted at 4 °C with 9% formic acid. The flow rate was 2.5 mL/h, and fractions of 0.8 mL were collected and assayed for radioactivity. (Lower frame) Optical density profile at 280 nm. (B) (Upper frame) CNBr fragments generated from activated phosphorylase *b* labeled either in the absence (solid line) or in the presence of 10 mM Glc-1-P (broken line) as described under Material and Methods. (Lower frame) Elution profile obtained from optical density measurements at 280 nm. Conditions for chromatography of these samples were the same as those described in (A). (C) (Upper frame) CNBr fragments generated from phosphorylase *b* (150 mg) labeled either in the absence of any effector (solid line) or in the presence of 10 mM 2′dAMP (broken line). (Lower frame) Elution profile obtained from optical density measurements at 280 nm. The column (2.2 × 90 cm) was eluted with 9% formic acid at 12.5 mL/h, in a cold room. Fractions of 4 mL were collected.

due to a modification of Arg-568? (2) In the case of phenylglyoxal inactivation, is the reactivity of Arg-568 also influenced by allosteric activators or inhibitors? In particular, is it reactive in phosphorylase *b* in the presence of activators and unreactive in their absence? To test these hypotheses, we used peptide maps to evaluate directly the extent of modification of Arg-568 without purifying peptide 563–572. Chymotryptic maps of CB15(−) from phosphorylase *a* have one major radioactive spot which is absent in maps of CB15(+). Purified fragment 563–572 runs at the same position as this spot.

(*a*) *Reactivity of Arg-568 with respect to Butanedione.* Phosphorylase *a* was modified by 2,3-butanedione in the presence of 0.5 mM AMP until 75% of the initial activity was lost. Excess of reagent was removed by filtration on a Sephadex G-25 column, and the modified enzyme (50 μM) was incubated with phenylglyoxal in the presence of 5′AMP. Under these conditions, the radioactivity incorporated into CB15 was reduced by 40% with respect to control samples untreated with butanedione, while the radioactivity incorporated into other CNBr peptides was unchanged. Autoradiography of the chymotryptic peptide maps obtained from CB15 showed that the major radioactive spot corresponding to fragment 563–572 was very faint in the butanedione-treated enzyme with respect to control enzyme. Thus, inactivation with butanedione prior to phenylglyoxal treatment specifically prevents modification of Arg-568.

(*b*) *Reactivity of Arg-568 in Phosphorylase b.* Phosphorylase *b* (50 μM) was incubated with phenylglyoxal in the presence of 1 mM AMP and 12% *tert*-butyl alcohol. We have shown previously (Dreyfus *et al.*, 1978) that under these conditions phosphorylase *b* adopts an "*a*-like" conformation. After isolation, the labeled enzyme was cleaved by CNBr and the peptide pool chromatographed on Sephadex G-50. The radioactive profile (Figure 1B) differed from that obtained with phosphorylase *a* (Figure 1A). However, when Glc-1-P (10 mM) was added to the modification medium, incorporation of [^{14}C]phenylglyoxal into the first peptide fraction (CB15) was reduced by 40%, as observed with phosphorylase *a* (Figure 1B). Simultaneously, the inactivation rate was reduced by 50–60%. Autoradiography of the chymotryptic peptide map of purified CB15(−) shows a major spot which migrates at the same position as the crucial peptide from phosphorylase *a* and is absent or very faint in maps derived from CB15(+). We conclude that, in activated phosphorylase *b*, arginine-568 is reactive toward phenylglyoxal modification and can be protected by Glc-1-P with simultaneous protection of the enzymatic activity, as in phosphorylase *a*.

Phosphorylase *b* (50 μM) in the absence of any ligand was incubated with phenylglyoxal (370 μM) until about 1 mol of reactant was incorporated per subunit (Table I). Modified phosphorylase was cleaved by CNBr, and the fragments generated were passed through a large Sephadex G-50 superfine column (Figure 1C). Although precise comparison with parts A and B of Figure 1 is difficult because of the different geometry of the columns used, it is clear that the relative radioactivity incorporated into CB15 was quite small, despite the absence of Glc-1-P in the reaction mixture. Furthermore, the chymotryptic map of CB15 does not exhibit the major radioactive spot which has been associated with peptide 563–572. This result shows directly that Arg-568 does not react with phenylglyoxal on an inactive form of phosphorylase.

(*c*) *Arginine Residues at the Nucleotide Binding Site.* Modification of phosphorylase with butanedione has provided evidence that arginine residues are also involved in the nucleotide binding site (Li et al., 1977; Dreyfus et al., 1980). A similar conclusion was obtained with phenylglyoxal. Thus,

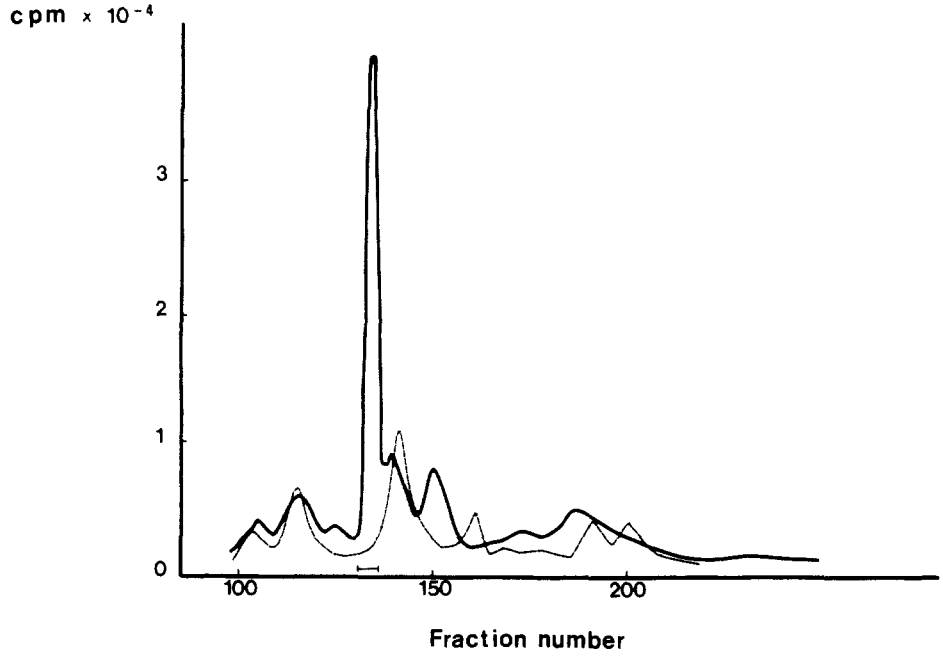

FIGURE 2: Separation of chymotryptic peptides from CB15 on Bio-Gel P4. CB15 fragments (2.8 μmol) were obtained from phosphorylase *a* labeled by [^{14}C]phenylglyoxal either in the absence of Glc-1-P (solid line) or in the presence of Glc-1-P (broken line). These fragments were digested with chymotrypsin, and the resulting peptide mixture was fractionated by gel filtration. The column (1.5 × 110 cm) of Bio-gel P4 was eluted with 9% formic acid at 6 mL/h, in a cold room. Fractions of 1.2 mL were collected and assayed for radioactivity.

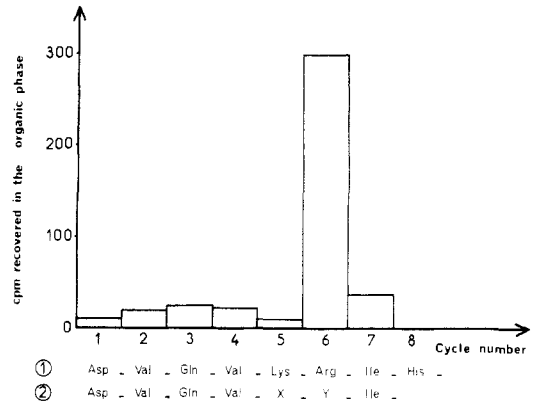

FIGURE 3: The modified 563–572 peptide is labeled at Arg-568. Modified 563–572 peptide (3.5 nmol, 800 cpm) was submitted to manual Edman degradation (Material and Methods). At each cycle, pooled *n*-butyl acetate extracts were counted for radioactivity. Before each new cycle, 8–10% of the material was removed and reacted with dansyl chloride for identification of the N-terminal residue, and the observed sequence is given. It was impossible by this method to distinguish between the acidic residues and their corresponding amides, but three turns of sequence using the method of Chang et al. (1978) on 2 nmol of starting material showed the N-terminal sequence to be Asp-Val-Gln (line 2). The sequence of peptide 563–572 obtained by Titani et al. (1977) is given (line 1).

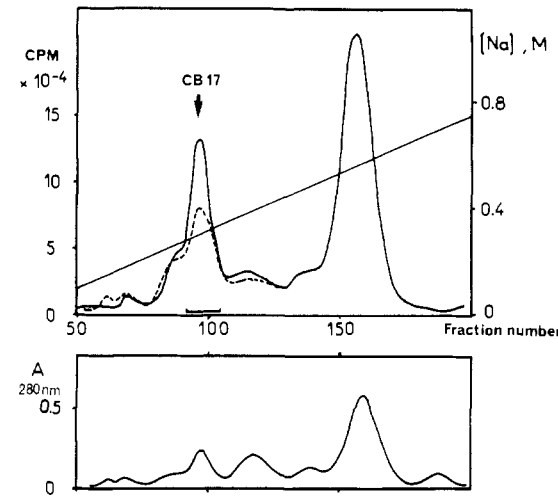

FIGURE 4: Protection from modification of one or several arginine residues located within CB17 (residues 242–349) by the binding of nucleotides to phosphorylase *b*. Of the fraction indicated by horizontal bar in Figure 1C, 250 mg was applied on a column (2.5 × 35 cm) of SP Sephadex C-25. Fragments were eluted at room temperature at 10 mL/h by a linear 800-mL gradient of 0.1 M sodium formate and 7 M urea, pH 2.9, to 0.75 M sodium formate and 7 M urea, pH 3.9. Fractions of 4 mL were collected. See Figure 1C for the identification of the symbols.

when phosphorylase *b* was modified in the presence of 10 mM 2′dAMP, an AMP analogue which only slightly activates the enzyme (Morange et al., 1976), the initial rate of phenylglyoxal incorporation was reduced by ~20% with respect to the uncomplexed enzyme (Table I). Simultaneously, the AMP-induced activity was protected. Sephadex G-50 chromatography of CNBr peptides showed that the presence of 2′dAMP during modification does not increase the radioactivity incorporated into CB15 and thus does not increase the reactivity of Arg-568 (Figure 1C). On the other hand, the amount of radioactivity incorporated in the second peak of the Sephadex G-50 elution profile was greatly reduced (Figure 1C). This fraction was resolved on a SP Sephadex C-25 column according to Koide et al. (1978). As shown in Figure 4, the arginine residues protected by 2′dAMP were all contained within the first peak in the elution profile, which correspond to CB17 (fragment 242–349) according to Koide et al. (1978). The identity of this peptide was confirmed by amino acid analysis (Table II). This fragment contains eight arginine residues. Since crystallographic work (Fletterick et al., 1979) has shown previously that the 5′phosphate group of nucleotides interacts with Arg-242, -308, and -309, which are all contained within this fragment, complete identification of these residues was not undertaken.

Discussion

A major aim of this work was the identification on the primary structure of phosphorylase of the arginine residues which are differentially labeled, depending upon the conformation—active or inactive—of the enzyme. Phenyl-

glyoxal was used here as an arginine-modifying reagent rather than 2,3-butanedione, which we used previously (Dreyfus et al., 1980) because arginine–butanedione complexes are very instable, making the isolation and characterization of labeled peptides difficult (Riordan, 1973).

In preliminary experiments, phosphorylase *a* was incubated with a 100–200 molar excess of modifying reagent, and we found that more than 10 mol of phenylglyoxal could be incorporated per mol of enzyme; thus an appreciable fraction of the 63 arginine residues of phosphorylase can be modified by phenylglyoxal, most of them being presumably unrelated to the catalytic site. A similar observation has been made by Li et al. (1977) on the basis of X-ray diffraction data on the butanedione-modified enzyme. The inactivation of the enzyme by phenylglyoxal proceeds much faster than the average modification rate of these residues and thus must be due to the modification of one (or one group of) highly reactive arginine residue(s). The substrate Glc-1-P offers an efficient protection, with a concomitant decrease in the initial rate of phenylglyoxal uptake. Within experimental error, we found that the protection of the enzyme activity provided by Glc-1-P, expressed as a fraction of the initial activity, is equal to the difference in molar incorporation of phenylglyoxal in the presence and absence of this substrate (cf. Table I). Assuming a 1:1 stoichiometry of the arginine–phenylglyoxal complex (see below), this means that glucose 1-phosphate protects only one arginine residue, the modification of which leads to complete inactivation of the enzyme. These findings were confirmed by peptide chemistry. Taking advantage of the strategy used by Titani et al. (1977) for the elucidation of the primary structure of phosphorylase, we were able to show that the reduced incorporation of phenylglyoxal observed in the presence of substrate was almost entirely due to protection of only one arginine residue, Arg-568. Essentially identical observations could be made with phosphorylase *b* modified in the presence of the activators AMP + alcohol. By contrast, in the absence of these activators, Arg-568 was not reactive in phosphorylase *b*, and the inactivation rate was slower (cf. below).

Although the influence of substrates and effectors on the inactivation rate of phosphorylase by phenylglyoxal and butanedione is similar, a major difference was that no conditions were found for which the enzyme could be completely protected against phenylglyoxal inactivation. Thus, in the presence of AMP and alcohol, phosphorylase *b* was inactivated by phenylglyoxal only 4 times faster than in the absence of effectors, while a factor of at least 50 was observed with butanedione. Saturating Glc-1-P concentrations reduced the rate of inactivation of phosphorylase *a* (or "activated" phosphorylase *b*) only by a factor of 2.5, instead of 5 for butanedione. Moreover, the addition of all substrates together, which virtually completely protects the enzyme against butanedione inactivation, is not more efficient than Glc-1-P alone in the case of phenylglyoxal. Conceivably, there is one (or one group of) residue(s) which cannot be protected by substrates and whose modification by phenylglyoxal inactivates the enzyme. This (these) residue(s) must be unreactive toward butanedione, or at least its modification does not affect the catalytic activity. On the other hand, Arg-568 modification by phenylglyoxal closely mimics the overall effect of butanedione on the activity of the enzyme in three respects: (a) it occurs only on the "activated" conformations of phosphorylase; (b) it causes inactivation of the enzyme; and (c) it can be prevented by the binding of substrates. On this basis, we conclude that the modification of Arg-568 is also responsible for butanedione inactivation. Supporting this view, we observed that, when phosphorylase *a* was 75% inactivated by butanedione prior to phenylglyoxal treatment, very little phenylglyoxal modification of Arg-568 is detected. By contrast, phenylglyoxal modification of other arginine residues was hardly diminished after pretreatment with butanedione, and this is taken to indicate that Arg-568 has an unusually high reactivity toward both butanedione and phenylglyoxal.

Twelve years after the first report of Takahashi (1968), the chemistry of the phenylglyoxal–arginine interaction is still poorly understood. It is generally assumed that phenylglyoxal reacts specifically with arginine to form a 2:1 complex, which is instable at basic pH but is stable in mild acid and does not regenerate arginine upon hydrolysis (Takahashi, 1968; Bond et al., 1980). On the other hand, evidence exists that in borate buffers, or at low phenylglyoxal concentration, the stoichiometry of the complex is, in fact, 1:1 (Borders & Riordan, 1975; Werber et al., 1975; Kazarinoff & Snell, 1976; Philips et al., 1979). In the present work, we found that purified 563–572 peptide contains only 1 mol of phenylglyoxal/mol. It is unlikely that the labeled peptide has fortuitously copurified with unlabeled 563–572 material since phenylglyoxal modification presumably alters both the charge (Takahashi, 1968) and the hydrophobicity of the modified peptide. Thus, the observed stoichiometry must reflect a 1:1 stoichiometry of the arginine–phenylglyoxal interaction. Moreover, there is no reason to believe that the stoichiometry may have changed from 2:1 to 1:1 during the purification procedure since at each step very little radioactivity appeared in low molecular weight fractions. Moreover, the 563–572 labeled peptide either from a total phosphorylase *a* chymotryptic digest or from a purified preparation comigrates on peptide maps, thus ruling out structural modification during purification. Therefore, we conclude that the initial stoichiometry of the arginine–phenylglyoxal complex must also have been 1:1. Presumably, the initial product of phenylglyoxal reaction on arginine, a cis diol, can be stabilized by borate, thus preventing the condensation of another molecule of reagent (cf., e.g., Kazarinoff & Snell (1976); Weber et al., 1975). In contrast with the 2:1 complex, the 1:1 complex is stable enough to allow conventional peptide chemistry to be done without a major problem. In particular, labeled peptides survive in the weakly basic media required for chymotryptic digestion, coupling to isothiocyanate during Edman degradation or cysteine alkylation in the presence of 6 M guanidinium chloride. The last result is of special interest, because in these experiments the guanidinium ion, an arginine analogue, is present in a 1000-fold molar excess over arginine residues; this makes unlikely, at least under our experimental conditions, the recently suggested possibility that phenylglyoxal may exchange between arginine residues under weakly basic conditions (Bond et al., 1980). On the other hand, we have repeatedly observed significant regeneration of arginine during acid hydrolysis, even in the presence of reducing agents (Table II). Other reports have described extensive regeneration of arginine from its complexes with cyclohexanedione (Patthy & Smith, 1975), 4-hydroxy-3-nitrophenylglyoxal (Borders et al., 1979), and phenylglyoxal itself (R. Kassab, personal communication). Further chemical work is clearly required to characterize completely the properties of the phenylglyoxal–arginine complex in proteins.

The conclusion that Arg-568 is close to the Glc-1-P binding site at the active site of the enzyme is supported by three-dimensional structure data recently obtained with phosphorylase *a* crystals grown in the presence of glucose (Fletterick & Madsen, 1980) and with phosphorylase *b* crystals grown

in the presence of IMP (Johnson et al., 1980). The situation of Arg-568 at or near the anion binding site at the active site of the enzyme is presumably responsible for its unusually high reactivity toward α-dicarbonyl reagents in the R conformation (Patthy & Thesz, 1980). In this respect, it is worthwhile to note that, on the primary structure, arginine-568 is located within a cluster of positively charged residues (Lys-567, Arg-568, His-570, Lys-573, and Arg-574), several of which have been shown to be located at or near the active site (Shimomura et al., 1978; Johnson et al., 1980), which presumably help to increase the reactivity of Arg-568 by promoting its deprotonation (Patthy & Thesz, 1980). In the present state of X-ray diffraction studies of phosphorylase, it is also possible to speculate on the origin of the lowered reactivity of Arg-568 in the T, compared to the R, conformation. In the crystal of phosphorylase *a* (T form), Arg-568 is hydrogen bounded to Asp-283 (N. B. Madsen and R. J. Fletterick, personal communication), which would presumably protect it from modifying reagents. On the other hand, Asp-283 is included in a segment of polypeptide chain which seems to move away during the T to R transition (Madsen et al., 1978). Conceivably this would deshield Arg-568, thus making it more reactive toward chemical modification and also available for substrate binding or for some other, yet undefined, functional role in the active site of phosphorylase.

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