Amino Acid Sequence at the Citrate Allosteric Site of Rabbit Muscle Phosphofructokinase†

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ABSTRACT: Previously, this laboratory has demonstrated [Colombo, G., & Kemp, R. G. (1976) *Biochemistry 15*, 1774–1780] that under appropriate conditions the citrate inhibitory binding site of rabbit skeletal muscle phosphofructokinase can be covalently modified by using pyridoxal phosphate and sodium borohydride. In the current study, phosphofructokinase was modified by [^{3}H]pyridoxal phosphate and sodium borohydride with or without the addition of citrate to protect the ligand binding site. The modified proteins were digested with trypsin, and the peptides were separated by high-pressure liquid chromatography. A comparison of the tryptic chromatographic profiles showed that while the label was broadly distributed among nine peaks in the elution profile of the enzyme modified in the presence of the protective ligand, a single peptide contained 70% of the total radioactivity of the enzyme modified in the absence of citrate. This peptide was presumed to contain at least part of the citrate inhibitory site of the enzyme. The sequence of the peptide was determined and shown to match with positions 528–536 of phosphofructokinase with the modified residue being Lys-529. A comparison of the sequence with that of procaryotic phosphofructokinase indicated that a homologous residue in the enzyme from *Bacillus stearothermophilis* is critical to an allosteric site. A second peptide that was the most abundant labeled peptide in the digest of the enzyme modified in the presence of citrate was found to be identical with the second most abundant peptide of the digest from the unprotected enzyme. This peptide corresponded to residues 681–692 with the lysine at position 684 being the site of phosphopyridoxylation. This sequence overlaps a peptide previously isolated from sheep heart phosphofructokinase that was identified as an AMP binding site [Weng, L., Heinrikson, R. L., & Mansour, T. E. (1980) *J. Biol. Chem. 255*, 1492–1496].

Mammalian phosphofructokinase has complex regulatory properties that are mediated by the interaction of allosteric ligands with a number of distinct binding sites [see Uyeda (1979) or Kemp and Foe (1983) for a review]. One of these allosteric ligands is citrate, which acts as a potent allosteric inhibitor of the enzyme. Previous work from this laboratory (Colombo & Kemp, 1976) described conditions for achieving a covalent modification of the citrate binding site using pyridoxal phosphate plus sodium borohydride. That the modification was at the citrate site was indicated by the observations that citrate protected against the activity loss accompanying modification and that the activity loss was the result of a greatly increased sensitivity of the enzyme to MgATP inhibition, consistent with the established synergism between MgATP and citrate. The modification eliminated citrate binding by the enzyme but enhanced MgATP binding.

In the current work, the peptide bearing the pyridoxal phosphate modification site was identified, and the sequence was compared to the previously identified allosteric binding site of procaryotic phosphofructokinase (Evans & Hudson, 1979). A peptide that represents a secondary modification site has been identified and was found to be immediately adjacent to a previously identified AMP binding site of phosphofructokinase (Weng et al., 1980).

Materials and Methods

Enzyme. Phosphofructokinase was prepared from fresh rabbit muscle as described by Kemp (1975). Second crystals were collected by centrifugation and resuspended in buffer at pH 7.0 consisting of 25 mM sodium β-glycerophosphate, 25 mM glycylglycine, 1 mM ethylenediaminetetraacetic acid (EDTA),[1] and 0.1 mM fructose-1,6-P_2. Removal of low molecular weight contaminants was achieved by gel exclusion chromatography over Sephacryl S-200 or Sepharose CL-6B equilibrated with the above buffer.

Assay of Phosphofructokinase Activity. The activity of both native and modified phosphofructokinase was assayed at 30 °C using a recording spectrophotometer. The assay at pH 8.0 contained 50 mM Tris–sulfate, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM NADH, 1 mM ATP, and 1 mM fructose-6-P. Auxiliary enzymes, consisting of 0.6 unit of aldolase and 0.3 units each of triosephosphate isomerase and α-glycerophosphate dehydrogenase, were dialyzed against 50 mM NaTes (pH 7.0) before use. Reactions were initiated by the addition of fructose-6-P.

For the assays at pH 7.1 to monitor the relative ATP inhibition of native and modified enzyme, the following conditions were used. The assay medium at 30 °C contained 50 mM NaTes, 0.1 mM EDTA, 6 mM $MgCl_2$, 0.15 M KCl, 0.1 mM dithiothreitol, 0.2 mM NADH, the indicated concentrations of ATP, and auxiliary enzymes as described above. The assay was started by the addition of fructose-6-P added to a final concentration of 1 mM.

Preparation of [^{3}H]Pyridoxal Phosphate. [^{3}H]Pyridoxal phosphate was prepared by a minor modification of the method

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[1] Abbreviations: Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; HPLC, high-pressure liquid chromatography; PFK, phosphofructokinase; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PTH, phenylthiohydantoin; kDa, kilodalton(s).

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of Stock et al. (1967). To 25 mg of pyridoxal phosphate (free-acid form) was added 300 L of H_2O, and then sufficient solid $NaHCO_3$ was added to bring the pH to 7.5–8.0. The mixture was cooled to 0 °C in an ice bath, and 10 mCi (0.014 mmol) of NaB^3H_4 was added. This reaction was stirred for 15 min at 0 °C and allowed to stand at 20 °C for 15 min, and 0.6 mg of unlabeled $NaBH_4$ was added. The mixture was cooled to 4 °C and adjusted to 4 M $HClO_4$ with 11.6 M $HClO_4$. Freshly prepared MnO_2 was then added, and the mixture was incubated with stirring for 15 h at −10 to −12 °C. Unconsumed MnO_2 was removed by centrifugation. Two milliliters of H_2O was added to the supernatant, followed by 1.5 mL of 2.0 M $KHCO_3$ with stirring at 0 °C. The pH was brought to 6.5 with KOH, and the $KClO_4$ was removed by centrifugation.

The supernatant was applied to a 1.0 cm × 25 cm column of Dowex 1-X8 (acetate). The column was washed with H_2O and then eluted with a gradient composed of 200 mL of H_2O in the mixing chamber and 200 mL of 5 M CH_3COOH in the reservoir. The major radiolabeled fraction was frozen and lyophilized. An aliquot of the pool was subjected to thin-layer chromatography on silica gel G plates and developed with dioxane/H_2O (7:3). A single yellow radioactive spot which corresponded to pyridoxal phosphate was seen following development, with an R_f of 0.48. The [^{3}H]pyridoxal phosphate, which had a specific activity of 1.2×10^3 cpm/nmol, was stored frozen and repurified by reverse-phase HPLC prior to use in modification experiments.

Phosphopyridoxylation of Phosphofructokinase. The enzyme was pyridoxylated under conditions adapted from Colombo and Kemp (1976). Phosphofructokinase at a concentration of 2–3 mg/mL was incubated in 50 mM Tes, 1.0 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM ATP (pH 7.0). After being cooled to 4 °C in an ice bath, the solution was made 1.0 mM with respect to Mg^{2+} by the addition of $MgSO_4$ and, when desired, 1 mM citrate. This solution was then equilibrated for 10 min, and the indicated concentration of [^{3}H]pyridoxal phosphate was added. The solution was incubated with stirring for 5 min. After the addition of 50–300 μL of 1-octanol, the reaction was terminated by the addition of 0.1–0.2 mL of sodium borohydride (10 mg/mL, 0.01 N NaOH). The enzyme was dialyzed overnight in the 50 mM Tes buffer described above. Excess radioactive reagent was removed by chromatography on a G-50 Sephadex column equilibrated with the same buffer. The extent of incorporation was determined either by the absorbance at 325 nm using the millimolar extinction coefficient of 10.2 for *N*-(phosphopyridoxal)lysine (Fischer et al., 1963) or by scintillation counting of the protein. Incorporation of radioactivity was followed by placing a 50-μL aliquot into 1 mL of 10% trichloroacetic acid and collecting the precipitate on a glass fiber filter disk by filtration. The filter disk was washed with 15 mL of 10% trichloroacetic acid and counted by liquid scintillation. Counting efficiencies of precipitates were determined by comparing the counts of soluble modified enzyme with those obtained by filtering an acid precipitate. Incorporation data are expressed relative to a protomer molecular weight of 83 000.

Tryptic Digestion and Peptide Separation Techniques. To identify specific residues modified after modification, the following procedure was employed. The enzyme was precipitated with ammonium sulfate at 60% saturation and redissolved in 6 M guanidine hydrochloride/60 mM *N*-ethylmorpholine acetate at pH 8.2. Unlabeled iodoacetate was added to a final concentration of 50 mM. Tryptic digestion was carried out with carboxymethylated enzyme after extensive dialysis against 100 mM ammonium bicarbonate and 2 mM dithiothreitol. The digestion was performed at 38 °C for 17 h with 2% (w/w) trypsin (Worthington). Peptides were isolated on a Supelco LC-3DP column (25 cm × 4.6 mm) using an acetonitrile gradient with either 0.1% trifluoroacetic acid or 0.1% trifluoroacetic acid plus 0.05% triethylamine as the mobile phase modifier. Elution was monitored by radioactivity and by the absorbance at 325 nm. Amino acid analysis was performed either with ninhydrin detection on a Glenco modular amino acid analyzer or by reverse-phase HPLC after PITC derivatization as described in protocols developed by Waters. Amino acid sequences were determined on an Applied Biosystems gas phase sequencer. PTH–amino acids were identified by HPLC on an Altex Ultrasphere ODS column using a variation of the method described in the manufacturer's manual.

Results and Discussion

General Strategy. Earlier data from this laboratory provided evidence that pyridoxal phosphate plus sodium borohydride modified muscle phosphofructokinase to produce an enzyme that appeared to be modified at the citrate inhibitory site (Colombo & Kemp, 1976). The rationale for the use of the reagent was that the citrate binding site is likely to have multiple positive charges which could provide for the interaction of pyridoxal phosphate and subsequent Schiff base formation if one or more of the positive charges is provided by an amino group. Evidence that the modification was indeed at the citrate site and produced changes similar to that seen with site occupancy by citrate was as follows. Dramatic changes in activity and equilibrium binding of ligand occurred upon the covalent binding of a single mole of reagent per mole of protomer, and these changes were protected against by the presence of citrate and MgATP. The modification greatly increased sensitivity to ATP inhibition, which is consistent with the observed synergism between MgATP and citrate (Colombo et al., 1975). Modification by pyridoxal phosphate and sodium borohydride abolished the binding of citrate but enhanced the binding of MgATP, again in concert with the observations of ATP–citrate synergism. These data suggested that a peptide bearing a phosphopyridoxylated lysine should be isolatable. To facilitate these studies, [^{3}H]pyridoxal phosphate was prepared as described under Materials and Methods. To localize the critical binding residue, rabbit skeletal muscle phosphofructokinase was reacted with [^{3}H]pyridoxal phosphate in the presence and in the absence of citrate. Earlier studies showed (Colombo & Kemp, 1976) that the enzyme protected by citrate incorporated pyridoxal phosphate but with no significant modification of activity or regulatory properties. A comparison of the tryptic peptide maps of protein labeled under protected and unprotected conditions should reveal the peptide(s) involved in the binding of citrate.

Effect of Phosphopyridoxylation on Activity. Muscle phosphofructokinase was incubated with varying concentrations of [^{3}H]pyridoxal phosphate as described under Materials and Methods. The extent of incorporation was determined both by the OD at 325 nm and by the amount of protein-bound radioactivity. These data are presented in Table I. Each modified preparation along with the control was assayed at three concentrations of ATP: 0.2, 1.0, and 2.5 mM. Previous assays of native enzyme had indicated that the lowest concentration was suboptimal, 1.0 mM was near-optimal, and the highest concentration was inhibitory. The data in Table I show that the phosphopyridoxylated enzyme had a much greater sensitivity to ATP inhibition with almost total inhibition seen

Table I: Effect of Phosphopyridoxylation on Activity[a]

pyridoxal phosphate (mM)	mol incorporated/ mol of protomer	activity (units/mg) in the presence of 0.2 mM ATP	1.0 mM ATP	2.5 mM ATP
0	0	86	108	41
0.5	0.66	28	4	0
1.0	0.97	30	2	0
2.5	1.40	27	1	0

[a] Phosphofructokinase was incubated with the indicated concentration of tritiated pyridoxal phosphate, in the absence of citrate, and other conditions indicated under Materials and Methods. After addition of $NaBH_4$, the enzyme was dialyzed, and the incorporation was determined by the absorbance at 325 nm and by scintillation counting. The modified enzymes were assayed at three concentrations of ATP with the pH 7.1 assay conditions described under Materials and Methods.

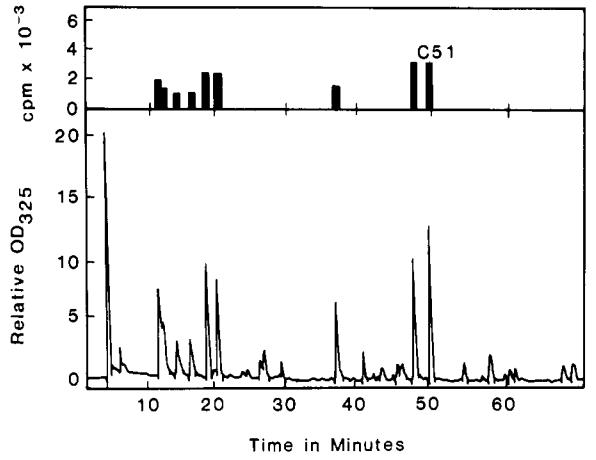

FIGURE 1: HPLC profile of a tryptic digest of phosphofructokinase phosphopyridoxylated in the presence of citrate. Phosphopyridoxylation was carried out as described under Materials and Methods. The content of pyridoxyllysine as determined by the OD at 325 nm and by radioactivity measurements was 1.0 mol/mol of protomer. The protein was carboxymethylated, digested with trypsin, and subjected to HPLC separation as indicated under Materials and Methods. The lower graph shows the OD at 325 nm and the upper graph indicates the level of radioactivity in HPLC fractions.

at 1.0 mM ATP. These data are in excellent agreement with the earlier data of Colombo and Kemp (1976) and with the prediction that the citrate site, known to be synergistic with ATP, was occupied by pyridoxyl phosphate.

Tryptic Digests and Chromatography of Phosphopyridoxylated Phosphofructokinase. Phosphofructokinase was incubated with 1 mM [^{3}H]pyridoxal phosphate with or without 1 mM citrate and other conditions as indicated under Materials and Methods. The enzyme modified in the presence of citrate incorporated 1.0 mol/mol and retained full activity when assayed under V_{max} conditions (pH 8.0). The enzyme modified in the absence of citrate incorporated 1.4 mol/mol of protomer and had lost 75% of its total activity. These results are consistent with the earlier data of Colombo and Kemp (1976). After reduction, carboxymethylation, and tryptic digestion, the peptides were separated by reverse-phase HPLC. The results with enzyme reacted in the presence of 1 mM citrate are described in Figure 1. The elution was monitored both by radioactivity and by absorption at 325 nm, which is characteristic of (phosphopyridoxyl)lysine. It can be seen that most of the absorption peaks also contained radioactivity. The radioactivity was fairly evenly distributed among nine peaks, and only one was taken for sequence analysis: the peak that eluted at 51 min contained approximately 20% of the total radioactivity and had the highest absorption at 325 nm. The

Table II: Edman Degradation of Pyridoxylated Peptides[a]

C51		M17		M21		M51	
amino acid	pmol	amino acid	pmol	amino acid	pmol	amino acid	pmol
M	195	I	279	I	388	M	178
G	136	K	0	K	0	G	123
A	232	Q	215	Q	255	A	207
K	1	S	14	S	215	K	0
A	154	A	173	A	474	A	140
M	68	A	173	A	459	M	55
N	60	G	112	G	176	N	51
W	25	T	18	T	99	W	61
M	39	K	29	K	3	M	36
A	48					A	37
G	35					G	26
K	219					K	229

[a] Peptide designations at the top of the columns refer to Figures 1 and 2. Yields indicated are for PTH–amino acids quantitated as indicated under Materials and Methods. The presence of lysine in the fourth cycle of peptides C51 and M51 and in the second cycle of M17 and M21 is assigned on the basis of established sequences (Poorman et al., 1984).

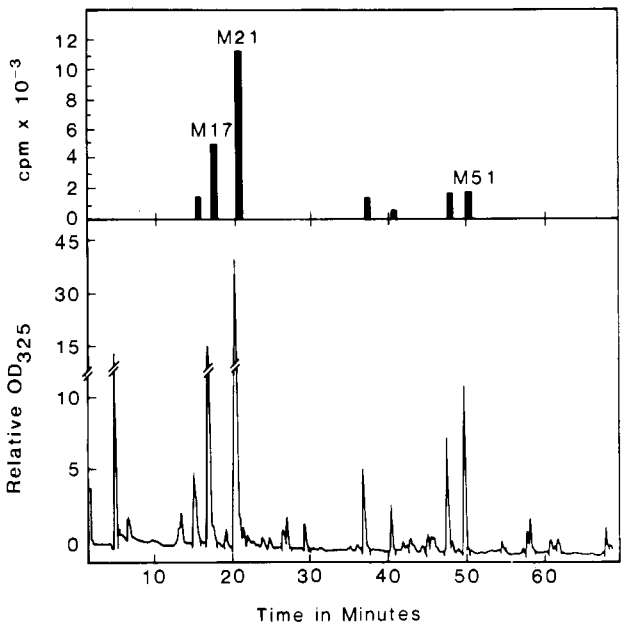

FIGURE 2: HPLC profile of a tryptic digest of phosphofructokinase phosphopyridoxylated in the absence of citrate. The (phosphopyridoxyl)lysine content of the modified protein was 1.4 mol/mol of protomer. See legend to Figure 1 for other details.

sequence of this peak, which is designated C51, is given in Table II.

The tryptic pattern of the enzyme not protected by citrate and thus presumably labeled at the citrate binding site is shown in Figure 2. Again, a peak of radioactivity, designated M51, emerged at 51 min, but in this instance, it contained only 7% of the total radioactivity. The analysis of peak M51 given in Table II show that it is identical with C51. On the other hand, most of the radioactivity was found in two peaks designated M17 and M21. The peak emerging at 17 min contained 19% of the total radioactivity while that eluting at 21 min had 51% of the total. The sequence analysis of the peaks are given in Table II. It can be seen that the sequences are identical, indicating that the incorporation into this particular peptide constitutes 70% of the total. One can reasonably assume that this peptide contains residues important in the citrate binding site of phosphofructokinase. On the basis of the sequence of the rabbit muscle enzyme (Poorman et al., 1984), lysine would be expected at the second cycle of peptides M17 and M21. Its absence and the absence of any identifiable PTH–amino

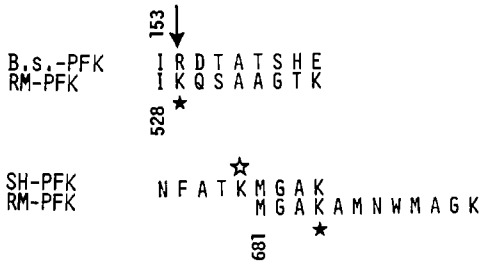

FIGURE 3: Comparison of identified phosphopyridoxylated sequences with those of other phosphofructokinases. The upper sequences compare the sequence of M19 and M21 (RM-PFK) with the homologous sequence from *B. stearothermophilis* (B.s.-PFK). The arrow indicates an Arg residue implicated in the allosteric binding site of the bacterial enzyme (Evans & Hudson, 1979). The solid star represents the modified residue identified in the current study. The lower two sequences compare the peptide at the AMP site of sheep heart phosphofructokinase (SH-PFK) (Weng et al., 1980) and peptides M51 and C51. The open star marks the residue modified by [(fluorosulfonyl)benzoyl]adenosine, and the solid star designates the lysine modified in the current study.

acid in the Edman degradation strongly suggest this position as the lysine that is modified in the phosphopyridoxylation. A modified lysine was of course originally predicted on the basis of the absorption spectra and on the failure of trypsin to cleave at the predicted lysine position. It should be noted that the citrate site was probably completely modified when one considers that 70% of 1.4 mol/mol suggests 1 mol of reagent incorporated into the specific site of the protomer. Although the total incorporation of label into the two protein preparations was not vastly different (1.4 vs. 1.0), the results show that the pattern of incorporation was quite different. This may be attributed to the enzyme being in two different conformations in the presence and absence of the inhibitor citrate.

It is curious that M17 and M21 gave identical sequences despite different elution times. One explanation would be that one of the peptides, probably M17, had an undetected arginyl residue following the terminal lysine, as predicted by the published sequence (Poorman et al., 1984). We occasionally have trouble detecting terminal arginines due to incomplete extraction from the membrane.

Relationships to Other Phosphofructokinases. On the basis of the crystal structure of phosphofructokinase from the procaryote *Bacillus stearothermophilis*, Evans et al. (1981) have proposed residues important in the binding of substrates and allosteric effectors. We (Poorman et al., 1984) have suggested that because of the close homology of the amino- and carboxyl-terminal halves of mammalian phosphofructokinase with procaryotic phosphofructokinase that the 80–84-kDa mammalian enzyme has evolved from the 35-kDa bacterial enzyme by gene duplication, fusion, and mutation of duplicated catalytic and regulatory sites to generate additional allosteric sites. The homology between the two halves and the bacterial enzyme is greater than 40% and is much higher than that in the regions of ligand binding sites. The upper part of Figure 3 compares the sequence of the citrate-interacting lysine of the rabbit muscle enzyme with the homologous sequence of *B. stearothermophilis* phosphofructokinase (Poorman et al., 1984). The sequence of M17 and M21 matches with the sequence at positions 528–536 previously identified (Poorman et al., 1984), which is homologous to *B. stearothermophilis* sequence 153–161. The Arg at position 154 of the bacterial enzyme has been implicated by crystallography to be important in the allosteric binding site (Evans et al., 1981). This residue is homologous to the lysine phosphopyridoxylated in the current study, which suggests as predicted by Poorman et al. that this allosteric site of the bacterial enzyme has evolved into one of the allosteric binding sites of rabbit skeletal muscle phosphofructokinase. There is one inconsistency in the sequence with that previously published by Poorman et al. (1984). This is at the assigned position 531 where Ile was previously identified. As indicated, the residue was found to be Ser, which is also in agreement with the unpublished genomic DNA sequence (S. Chang, personal communication).

The second peptide that was sequenced (C51 and M51) was present in tryptic digests of both protected and unprotected modified enzymes. Thus, this peptide is not likely a component of the citrate site but does represent an exposed region of the molecule with multiple positive charges. There are likely to be several regions with these properties because they may represent binding sites for phosphate-bearing ligands. Phosphofructokinase has three sites for nucleotide phosphates, two sites for sugar phosphates, and an undetermined number of binding sites for inorganic phosphate (Kemp & Foe, 1983). As shown in the lower part of Figure 3, the labeled peptide lines up with a tryptic peptide from sheep heart phosphofructokinase that was labeled by [(fluorosulfonyl)benzoyl]-adenosine at what appears to be the AMP allosteric site (Weng et al., 1980). The labeled lysine of the sheep heart enzyme was located four residues from the site of phosphopyridoxylation of the rabbit muscle enzyme. It is likely that the basic residues at positions 680 and 684 of the rabbit enzyme are also involved in a nucleoside phosphate binding site.

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