Role of the N-terminal region in covalent modification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: comparison of phosphorylation and ADP-ribosylation

Jose Luis ROSA,* J. Xavier PÉREZ,* Francesc VENTURA,* Albert TAULER,* Joan GIL,* Makoto SHIMOYAMA,† Simon J. PILKIS‡ and Ramon BARTRONS*§

*Unitat de Bioquímica, Departament de Ciències Fisiològiques Humanes i de la Nutrició, Universitat de Barcelona, Campus de Bellvitge, 08907-Hospitalet de Llobregat, Spain, †Department of Biochemistry, Shimane Medical University, Izumo 693, Japan, and ‡Department of Biochemistry, Medical School, University of Minnesota, Minneapolis, MN 55455-037, U.S.A.

The effect of cyclic AMP (cAMP)-dependent phosphorylation and ADP-ribosylation on the activities of the rat liver bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2), was investigated in order to determine the role of the N-terminus in covalent modification of the enzyme. The bifunctional enzyme was demonstrated to be a substrate *in vitro* for arginine-specific ADP-ribosyltransferase: 2 mol of ADP-ribose was incorporated per mol of subunit. The K_m values for NAD⁺ and PFK-2/FBPase-2 were 14 μ M and 0.4 μ M respectively. A synthetic peptide (Val-Leu-Gln-Arg-Arg-Arg-Gly-Ser-Ser-Ile-Pro-Gln) corresponding to the site phosphorylated by cAMP-dependent protein kinase was ADP-ribosylated on all three arginine residues. Analysis of ADP-ribosylation of analogue peptides containing only two arginine residues, with the third replaced by alanine, revealed that ADP-ribosylation occurred predominantly on the two most C-terminal arginine residues. Sequencing of the ADP-ribosylated native

enzyme also demonstrated that the preferred sites were at Arg-29 and Arg-30, which are just N-terminal to Ser-32, whose phosphorylation is catalysed by cAMP-dependent protein kinase (PKA). ADP-ribosylation was independent of the phosphorylation state of the enzyme. Furthermore, ADP-ribosylation of the enzyme decreased its recognition by liver-specific anti-bifunctional-enzyme antibodies directed to its unique N-terminal region. ADP-ribosylation of PFK-2/FBPase-2 blocked its phosphorylation by PKA, and decreased its PFK-2 activity, but did not alter FBPase-2 activity. In contrast, cAMP-dependent phosphorylation inhibited the kinase and activated the bisphosphatase. These results demonstrate that ADP-ribosylation of arginine residues just N-terminal to the site phosphorylated by PKA modulate PFK-2 activity by an electrostatic and/or steric mechanism which does not involve uncoupling of N- and C-terminal interactions as seen with cAMP-dependent phosphorylation.

INTRODUCTION

6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) catalyses both the synthesis and degradation of fructose 2,6-bisphosphate (Fru-2,6- P_2), an allosteric activator of 6-phosphofructo-1-kinase and an inhibitor of fructose-1,6-bisphosphatase [1–3]. The liver enzyme consists of two identical subunits of 55 kDa, each containing two active sites, corresponding to the kinase and bisphosphatase activities. Phosphorylation of Ser-32 of liver PFK-2/FBPase-2 by cyclic AMP (cAMP)-dependent protein kinase (PKA) results in kinase inactivation and bisphosphatase activation, which explains the ability of glucagon to decrease Fru-2,6- P_2 levels in liver. The bifunctional enzyme integrates a number of metabolic and hormonal signals by means of allosteric effectors and phosphorylation/dephosphorylation processes, and by regulation of its gene expression. PFK-2/FBPase-2 thus provides an important switching mechanism between glycolysis and gluconeogenesis in mammalian liver.

Recent evidence supports the hypothesis that the N- and C-terminal regions of the liver bifunctional enzyme interact individually with the kinase and bisphosphatase domains, respectively [4]. The bifunctional-enzyme homodimer has been postulated to exist in an antiparallel configuration, with the N-

and C-terminal regions interacting separately with the active sites of their respective domains, as well as interacting with the opposing terminus in the other domain in both the phosphorylated and dephosphorylated states [5]. In support of this hypothesis, deletion of either the N- or C-terminal regions simultaneously leads to disruption of the N- and C-terminal interactions, resulting in a 5–10-fold activation of the bisphosphatase and an inhibition of the kinase [6]. PKA-catalysed phosphorylation of Ser-32 causes an inhibition of the kinase and 2–4-fold activation of the bisphosphatase and phosphorylation has also been postulated to act by uncoupling the N- and C-terminal interactions [4,5]. Another covalent modification in the N-terminal region of the enzyme, involving a group different from phosphate and with different charge, would provide an additional test for this hypothesis.

Mono(ADP-ribosyl)transferases (ADPRT) catalyse transfer of the ADP-ribose moiety from NAD⁺ to specific acceptors and are present in a large number of animal tissues. This post-translational protein modification is a mechanism by which living organisms modify the function of many important regulatory proteins [7,8]. There are several examples of proteins which are subject to covalent modification by both phosphorylation and ADP-ribosylation. For example, the enzyme from chicken peripheral polymorphonuclear cells [9]

Abbreviations used: PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); FBPase-2, fructose-2,6-bisphosphatase (EC 3.1.3.46); Fru-2,6- P_2 , fructose 2,6-bisphosphate; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase (EC 2.7.1.37); ADPRT, mono(ADP-ribosyl)transferase (EC 2.4.2.31); DTT, dithiothreitol.

§ To whom correspondence should be addressed, at: Unitat de Bioquímica, Facultat Odontologia, Universitat de Barcelona, Campus de Bellvitge, 08907-Hospitalet de Llobregat, Spain.

catalyses the ADP-ribosylation of arginine residues in two key enzymes of carbohydrate metabolism, L-pyruvate kinase [10] and phosphorylase kinase [11], and histone H1 [12] at an arginine next to the serine that is phosphorylated by PKA [9–12]. For pyruvate kinase, this leads to suppression of the subsequent phosphorylation of the enzyme. The Kemptide peptide (Leu¹-Arg-Arg-Ala-Ser-Leu-Gly⁷), an analogue peptide of the liver pyruvate kinase phosphorylation site and a substrate of PKA, has also been shown to be ADP-ribosylated at an arginine by this ADPRT [10]. High concentrations of this peptide suppress the phosphorylation of pyruvate kinase by PKA. However, although rat liver pyruvate kinase can be ADP-ribosylated, the enzyme activity is not affected [10].

Since PFK-2/FBPase-2 contains an amino acid sequence (Arg²⁸-Arg-Arg-Gly-Ser-Ser-Ile³⁴) [1] related to the phosphorylation and ADP-ribosylation sites of Kemptide peptide, phosphorylase kinase, histone H1 and L-pyruvate kinase, we investigated whether ADPRT could ADP-ribosylate purified liver PFK-2/FBPase-2 at a site(s) in the N-terminal region and how this modification affected the two activities of the enzyme.

EXPERIMENTAL

Materials

[γ -³²P]ATP (3000 Ci/mmol) and [adenylate-³²P]NAD⁺ (1000 Ci/mmol) were from Amersham. The catalytic subunit of PKA, thermolysin (protease type X), tosyl-L-phenylalanyl-chloromethane, tosyl-L-lysylchloromethane, soybean trypsin inhibitor and pyruvate kinase phosphorylation-site analogue peptide were obtained from Sigma. DEAE-Sephadex G-25 (superfine) was obtained from Pharmacia Fine Chemicals. Phosphocellulose (P-11) was from Whatman. Chemicals used for HPLC were from Burdick and Jackson Laboratories Inc. All other biochemical reagents were from Boehringer. All chemicals were of analytical grade.

Enzymes

ADPRT was purified from chicken peripheral polymorphonuclear cells [9] or from turkey erythrocytes [13]. Rat liver PFK-2/FBPase-2 was purified as described previously [14,15]. Purified turkey erythrocyte ADPRT was kindly given by Dr. Joel Moss (N.I.H., U.S.A.).

ADP-ribosylation of PFK-2/FBPase-2

For the assay of ADP-ribose incorporation, the reaction mixture contained 50 μ M [³²P]NAD⁺ (2000 c.p.m./pmol), 5 mM dithiothreitol (DTT), 50 mM Tris/HCl (pH 7.1), purified hepatic PFK-2/FBPase-2 (0.3 μ g) and ADPRT (36 ng) in a total volume of 20 μ l. The mixtures were incubated at 25 °C for 30 min, and the reaction was stopped with 1 ml of 10% trichloroacetic acid or with loading buffer [16]. After incubation, the samples were fractionated by SDS/PAGE (10% gel) [16] and the gel was subjected to autoradiography [17].

Preparation and purification of a ³²P-labelled thermolytic peptide of PFK-2/FBPase-2

PFK-2/FBPase-2 (0.5–2 mg; 1 mg/ml) was incubated at 30 °C with ADPRT in a reaction mixture containing 50 mM Tris/HCl, pH 7.1, 0.2 mM [³²P]NAD⁺ (2000 c.p.m./pmol), 4 mM MgCl₂, 0.1 mM EDTA and 0.1 mM DTT. Incorporation was monitored by the method of Witt and Roskoski [18]. After 15 min when the ³²P incorporation had reached a plateau (1.6–1.8 mol of ³²P/mol of subunit), proteolysis was initiated by addition of thermolysin

and CaCl₂ to give final concentrations of 1 mg/ml and 0.1 mM respectively. Before this addition, the thermolysin was incubated at 30 °C for 15 min with 20 μ g/ml each of soybean trypsin inhibitor, tosyl-L-phenylalanylchloromethane and tosyl-L-lysylchloromethane. The thermolytic digestion of PFK-2/FBPase-2 was allowed to proceed at 30 °C for 30 min. The reaction mixture was then diluted with 10 vol. of 20 mM triethylamine bicarbonate (pH 8.2)/1 mM EDTA and was immediately applied to a column (0.7 cm $\times$ 2 cm) of DEAE-Sephadex equilibrated with 20 mM triethylamine bicarbonate. The column was then washed with 20 mM triethylamine bicarbonate, and the [³²P]phosphopeptide emerged in the wash. The pooled ³²P-containing fractions were dried by rotary evaporation, redissolved in 20 mM ammonium acetate, pH 5.5, and subjected to gel filtration on a Sephadex G-25 superfine column (1 cm $\times$ 90 cm) that had been previously equilibrated with 20 mM ammonium acetate, pH 5.5. The flow rate was 15 ml/h. A single peak of ³²P was eluted at an elution-volume/void-volume ratio of 1.44. The fractions containing ³²P were applied directly to a column (0.8 cm $\times$ 4 cm) of phosphocellulose equilibrated with 20 mM ammonium acetate. The column was developed with a linear gradient of ammonium acetate from 20 to 250 mM, and the ³²P was eluted as one peak at a salt concentration of 200 mM. After rotary evaporation, the ³²P-containing fractions were dissolved in 0.1% trifluoroacetic acid and subjected to HPLC on a C₁₈ column. HPLC was performed in a Beckman 334 instrument with a Beckman Ultrasphere-ODS column. Buffer A was 0.1% trifluoroacetic acid, and buffer B was 0.1% trifluoroacetic acid/50% acetonitrile. The gradient used was: 0–10 min, 0% B (i.e. 100% A); 10–15 min, 0–15% B; 15–75 min, 15–45% B; 75–80 min, 45–100% B. The flow rate was 1 ml/min, and fractions (1 ml) were collected and counted for Čerenkov radiation.

Sequence analysis

Automated Edman degradations were performed with an Applied Biosystems model 470 A protein sequencer equipped with a model 120 on-line HPLC system for analysis of amino acid phenylthiohydantoin as specified by Applied Biosystems. Amino acid compositions of purified peptides were analysed by the method of Bidlingmeyer et al. [19].

Peptide synthesis

Peptides were manually synthesized by the solid-phase method on a 0.7-nmol scale by a previously described protocol [20,21]. In addition to Boc¹-Gln esterified to 1% cross-linked chloromethylated styrene divinylbenzene (0.47 nmol/g) and t-amyloxycarbonyl-L-Arg(N^ε-tosyl), the following Boc-L-amino acids were used: Val, Leu-H₂O, Gly (4-nitrophenyl ester), Ala, Ser (O-benzyl), Thr (O-benzyl), Ile, H₂O, Pro and Boc-Gly. Dicyclohexylcarbodi-imide was used as the coupling agent other than in the active ester coupling of glutamine. Completeness of coupling of each residue was assessed by the ninhydrin test [22]. Peptides were cleaved from the resin with anhydrous HF/anisole (9:1, v/v) for 30 min at 0 °C [23]. After removal of residual anisole with ethyl acetate, the peptide was extracted from the resin with 1 M acetic acid and immediately freeze-dried. The amino acid sequences of the peptides that were synthesized are shown in Table 1.

Purification and analysis of peptides

Crude cleavage peptides were passed over a column (2.0 cm $\times$ 95 cm) of Sephadex G-25 (medium grade) in 10% acetic acid. The peak of peptide material was detected by the

Sakaguchi reagent [24], pooled, and freeze-dried. Final purification of samples of each peptide was achieved by HPLC on a Perkin Elmer Series 4 chromatograph linked to an LC-75 spectrophotometer set at 210 nm, an LC1-100 computing integrator, and a LKB Redirac fraction collector. Reversed-phase HPLC was performed on a semi-preparative (1.0 cm $\times$ 25 cm) column of Vydak 218TP1010, containing 300 Å (30 nm)-pore-diameter 10 μ m C₁₈ bonded phase. Samples were run at room temperature at a flow rate of 5 ml/min at a column pressure of 6–7 MPa. Peptides were eluted with a mixture of solutions A and B, which contained 0.1 % trifluoroacetic acid in water and 0.1 % trifluoroacetic acid in acetonitrile respectively. The column was equilibrated in 5 % solvent B, and the elution gradient was: 0–2.5 min, 5 % B; 2.5–37.5 min, 5–30 % B (linear); 37.5–40.0 min, 30–70 % B (linear). The major peak was pooled and freeze-dried.

Purity of peptides was assessed by TLC on cellulose sheets in the previously described solvents [20]. All peptides were pure, as determined by ninhydrin staining. Stock solutions of peptides were further analysed and quantified by amino acid analysis of duplicate acid hydrolysates (5.7 M HCl, 110 °C, 22 h). Amino acid analysis was performed by the method of HPLC after pre-column derivative formation with phenylisothiocyanate as described by Heinrikson and Meredith [25].

ADP-ribosylation of peptides

ADP-ribosylation assays were conducted for 5 min at 30 °C in a reaction mixture (final volume 0.08 ml) containing 50 or 100 mM potassium phosphate buffer (pH 6.8), 2 mM MgCl₂, [³²P]NAD⁺ (75–170 c.p.m./pmol), peptide substrate as indicated, 3 mM DTT and either 0.04–0.4 μ g/ml catalytic subunit of PKA or 2–40 ng of ADPRT. For determination of kinetic constants, each peptide was used over an 8.3–10-fold range of concentrations around its apparent K_m value. The amount of ADPRT used depended on the preliminary V_{max} value of the particular peptide substrate. To determine the stoichiometry of peptide ADP-ribosylation, the above conditions were modified such that the reaction mixture contained 100 μ M peptide, 3 mM MgCl₂, 0.8 mM [³²P]NAD⁺, and either 0.2–2.0 μ g/ml catalytic subunit of PKA or 5–40 ng of ADPRT. All reactions were initiated by addition of enzyme, and ADP-ribose incorporation was determined by the phosphocellulose paper method [18] using a phosphoric acid procedure.

Kinetic constants were determined from initial-velocity data fitted to the Michaelis–Menten equation by the method of weighted least squares. Less than 15 % of the least abundant substrate was consumed, and reactions were linear with respect to time and amount of enzyme. Kinetic analyses were repeated at least three times.

ADP-ribosylation and phosphorylation of PFK-2/FBPase-2

ADP-ribosylation was performed as described for the standard assay, but with non-radioactive NAD⁺. After 30 min, the mixture was incubated in 100 mM Hepes (pH 7.1), 5 mM MgCl₂, 0.5 mM [γ -³²P]ATP-Mg (1000 c.p.m./pmol) and 2 m-units/ml catalytic subunit of PKA (Sigma) for 30 min at 30 °C, in a total volume of 25 μ l. The reaction was stopped with loading buffer, and the samples were fractionated by SDS/PAGE (10 % gel). The gels were subjected to fluorography or autoradiography [17].

Phosphorylation and ADP-ribosylation of PFK-2/FBPase-2

For the assay of phosphorylation, the reaction mixture contained 20 mM Hepes (pH 7.1), 5 mM MgCl₂, 1 mM DTT, 0.5 mM ATP-Mg, purified hepatic PFK-2/FBPase-2 (0.3 μ g) and 2 m-

units/ml catalytic subunit of PKA (Sigma) in a total volume of 20 μ l. The mixtures were incubated at 30 °C for 30 min. After incubation, 50 μ M [³²P]NAD⁺ (~2000 c.p.m./pmol), 5 mM DTT, 50 mM Tris/HCl (pH 7.1) and 36 ng of ADPRT were added, and the mixture was incubated for 30 min at 25 °C in a total volume of 25 μ l. The reaction was stopped by addition of electrophoresis buffer and the samples were fractionated by SDS/PAGE (10 % gel). The gels were subjected to fluorography or autoradiography.

Assay of PFK-2/FBPase-2 activities

PFK-2 activity was determined by a modification of the method described by Bartrons et al. [26]. Samples were incubated at 30 °C in 100 mM Hepes (pH 7.1), containing 100 mM KCl, 20 mM KF, 1 mM DTT, 5 mM P_i, 5 mM MgCl₂, 0.5 mM ATP-Mg, 0.1 mM fructose 6-phosphate and 0.3 mM glucose 6-phosphate. FBPase-2 activity was assayed by measuring the rate of hydrolysis of [2-³²P]Fru-2,6-P₂ to [³²P]P_i and fructose 6-phosphate, as described in [27,28]. The protein concentration was determined as described by Bradford [29], with bovine serum albumin as standard. One unit of enzyme activity catalyses the formation of 1 μ mol of product per min under the specified assay conditions.

Western-blot analysis

Immunoblot analysis was performed essentially as described by Burnette [30], with a 1:500 dilution of polyclonal antibody raised against rat liver PFK-2/FBPase-2 [28] (common antibody) or with a 1:200 dilution of polyclonal antibody raised against a synthetic decapeptide (GELTQTRLQK), corresponding to the N-terminus of rat liver PFK-2/FBPase-2 [31] (liver-specific antibody). The liver-specific antibody was kindly given by Dr. Louis Hue (Louvain University, Belgium). Bound antibodies were detected by incubation with ¹²⁵I-labelled Protein A [(1–2) $\times$ 10⁶ c.p.m./ml] for 30 min and, after washing, the blot was brought into contact with X-ray film. The amount of enzyme was evaluated by densitometric scanning of the autoradiograms with an LKB Ultrosan XL laser densitometer and GelScan XL (2.1) software.

RESULTS

Evidence for PFK-2/FBPase-2 ADP-ribosylation

Incubation of purified hepatic PFK-2/FBPase-2 with [³²P]NAD⁺ and ADPRT resulted in the labelling of the bifunctional enzyme.

Figure 1 ADPRT ADP-ribosylates hepatic PFK-2/FBPase-2 in a concentration-dependent manner

The ADP-ribosylation assay was performed as indicated in the Experimental section. Results of a representative experiment are shown in the insert.

Figure 2 Effect of pH and P_i on ADP-ribosylation of PFK-2/FBPase-2

ADP-ribosylation of PFK-2/FBPase-2 was performed at pH 7.0 (○, ●) or pH 9.0 (□, ■) with (●, ■) or without (○, □) 5 mM potassium phosphate.

The intensity of the labelled band was dose- and time-dependent (Figures 1 and 2). The apparent K_m values for NAD^+ and PFK-2/FBPase-2, determined by double-reciprocal plots, were 14 μM and 0.4 μM respectively.

Effect of pH and P_i on ADP-ribosylation of PFK-2/FBPase-2

Preliminary experiments indicated that the rate of ADP-ribosylation was optimal at pH values greater than 8.0 (results not shown), and pH 9.0 was chosen to ensure rapid modification of the enzyme. In order to determine whether the enzyme could be ADP-ribosylated under more physiological conditions, its rate of ADP-ribosylation by the erythrocyte ADPRT was tested at pH 7.0 and 9.0 in the absence and presence of P_i . Phosphate has been shown to be an important physiological allosteric effector of the bifunctional enzyme [2,32]. It enhances the affinity of the PFK-2/FBPase-2 for fructose 6-phosphate by 50-fold without changing the V_{max} , whereas it enhances the V_{max} of the FBPase reaction under conditions of saturating substrate. The effect of phosphate on the kinase reaction is greatest at low, physiological, pH values [2,32]. P_i probably acts by changing the conformation of the enzyme. Figure 2 shows that at pH 7.0 the initial rate of ADP-ribosylation was 15–20% of that at pH 9.0. After 60 min, about 2.0 mol of [^{32}P]NAD⁺ was incorporated per mol of subunit at pH 9.0, whereas at pH 7.0 only 0.4 mol of [^{32}P]NAD⁺ was incorporated and the rate was still linear. Addition of 5 mM phosphate increased the rate by 5–7-fold, and it reached a plateau of about 1.5 mol of ^{32}P /mol of enzyme subunit. The same rate and stoichiometry of ADP-ribosylation was observed at pH 9.0 in the presence of phosphate. In the absence of phosphate, the stoichiometry of ADP-ribosylation levelled off at about 2 mol of ADP-ribose/mol of subunit. The results demonstrate that ADP-ribosylation is favoured at high pH in the absence of phosphate, but that in its presence the rate is independent of pH from 7.0 to 9.0. Since phosphate had no effect on ADP-ribosylation of small arginine-containing peptides (results not shown), it is probably due to an effect on the conformation of the enzyme.

Table 1 Automated Edman degradation of the thermolytic peptide isolated from PFK-2/FBPase-2

The thermolytic peptide containing the site ADP-ribosylated by ADPRT was isolated from PFK-2/FBPase-2 as described in the Experimental section. The peptide was subjected to automated Edman degradation in a gas-phase (ABI740A) sequencer; the product of each cycle was counted for Čerenkov radiation and analysed for amino acid phenylthiohydantoin. A sample (2 nmol) of peptide was applied to the glass-fibre filter of the ABI470 instrument. A dash indicates no positive identification of phenylthiohydantoin in amino acids. The same sequence was obtained with two different preparations of enzyme. Yields of minor amino acid at each cycle are not shown.

Cycle	Residue	Amount (pmol)	Radioactivity (c.p.m.)
1	Val	1678	108
2	Leu	1558	162
3	Gln	1180	125
4	Arg	423	285
5	—	—	1101
6	—	—	2965
7	Gly	632	632
8	Ser	309	592
9	Ser	158	608
10	Ile	145	409
11	Pro	77	388
12	Gln	53	390

Site of ADP-ribosylation of liver PFK-2/FBPase-2

Since pyruvate kinase, a substrate for PKA, had been shown to be ADP-ribosylated at an arginine residue adjacent to the serine that is phosphorylated by PKA, it was decided to determine whether PFK-2/FBPase-2, also a substrate for this kinase, was also ADP-ribosylated at some arginine residue next to the phosphorylated serine. There had been a previous report of the isolation of a [^{32}P]phosphopeptide by brief incubation of the enzyme with thermolysin after its phosphorylation by PKA [33]. The incubation with thermolysin converted the enzyme into a form with a subunit molecular mass of about 35 kDa, which contains all the ^{32}P radioactivity located in the phosphorylation-site peptide (Val-Leu-Gln-Arg-Arg-Arg-Gly-Ser-Ser-Ile-Pro-Gln). This peptide could be purified by ion-exchange chromatography, gel filtration and reverse-phase HPLC, and sequenced. This same approach was used to isolate the ADP-ribosylated thermolytic [^{32}P]peptide for the enzyme. When PFK-2/FBPase-2 was incubated with [^{32}P]NAD⁺ and ADPRT, the bifunctional enzyme was labelled to a level of 2 mol of ^{32}P /mol of subunit. The ^{32}P -labelled peptide was purified as described for the [^{32}P]phosphopeptide and subjected to automated analysis using a gas-phase sequencer. Positive identification of all of the residues was obtained, with the exception of cycles 5 and 6, where no phenylthiohydantoin derivative was recovered (Table 1). The yield of Arg at cycle 4 was also low. Although all three arginine residues may be ADP-ribosylated, the preferred sites are clearly at Arg-29 and Arg-30. Since the arginine derivative is unstable to Edman degradation, its position in a sequence was routinely identified as a burst of ^{32}P at cycles 5 and 6. The appearance of a small amount of radioactivity at cycle 4 suggests ADP-ribosylation at Arg-28. The results indicate that the arginine residues in the phosphorylation-site region are the site of ADPRT-catalysed ADP-ribosylation of the native PFK-2/FBPase-2.

During the thermolysin digestion of the ADP-ribosylated bifunctional enzyme, nearly 90% of ^{32}P radioactivity was released as trichloroacetic-soluble radioactivity, which was subsequently

Table 2 Apparent kinetic constants of ADPRT for ADP-ribosylation of synthetic peptides

Kinetic constants were determined as described in the Experimental section, and the values are given as means $\pm$ S.E.M.

Peptide no.	Peptide sequence	Apparent K_m (μ M)	V_{max} (μ mol/min) per mg)
1	VLQRRRGSSIPQ	45 $\pm$ 15	3 $\pm$ 1
2	VLQARRGSSIPQ	63 $\pm$ 5	2.9 $\pm$ 0.8
3	VLQRRARGSSIPQ	72 $\pm$ 7	0.7 $\pm$ 0.2
4	VLQRRAGSSIPQ	150 $\pm$ 20	0.5 $\pm$ 0.1

recovered in the purified thermolytic peptide. To assess further the specificity of this modification, the separate bisphosphatase domain of the bifunctional enzyme was incubated with ADPRT and NAD⁺ under the same conditions as for the intact enzyme. Under these conditions the bisphosphatase domain, which does not contain any cAMP-dependent-phosphorylation-site sequences, was ADP-ribosylated to only about 0.12 mol of ³²P/mol of enzyme (results not shown). This ³²P incorporation probably represents some non-specific modification of arginine residues. Bisphosphatase activity was unaffected by this low level of ADP-ribosylation (results not shown).

ADP-ribosylation of phosphorylation-site peptides

The above results indicate that the arginines located at the phosphorylation site region, mainly Arg-29 and Arg-30, were ADP-ribosylated by ADPRT. In order to analyse the relative effect of these arginines on the extension of the ADP-ribosylation reaction, we designed several peptides containing the bifunctional-enzyme cAMP-dependent phosphorylation site sequence and with Arg replaced by Ala. The assay conditions for ADP-ribosylation were chosen as optimal for ADP-ribosylation of the parent peptide (Val-Leu-Gln-Arg-Arg-Arg-Gly-Ser-Ser-Ile-Pro-Gln) of the synthetic peptides shown in Table 2. Peptide 1 was readily ADP-ribosylated to about 3 mol of ³²P/mol of peptide (results not shown). Each of the analogue peptides with a single arginine-to-alanine replacement was also ADP-ribosylated to approx. 1.5–2.0 mol of ³²P/mol of peptide. The influence of various concentrations of each peptide on the initial velocity of ADP-ribosylation by ADPRT was determined, and the results are shown in Table 2. The parent peptide had a K_m of 45 μ M and a V_{max} of 3 μ mol/min per mg. This peptide is a substantially better substrate for PKA, with a low-micromolar K_m and a V_{max} of over 13 μ mol/min per mg for Ser-32 phosphorylation. Peptide 2, which had two instead of three arginine residues, with the most N-terminal residue replaced by Ala, had essentially identical V_{max} and K_m values for ADP-ribosylation compared with peptide 1. Peptide 3, which had the second Arg replaced by Ala, had a V_{max} only 23 % of that of the parent peptide, whereas its K_m was similar to that of peptide 2. Peptide 4 was ADP-ribosylated with a V_{max} only 17 % of that of the parent peptide, with a K_m nearly 3-fold higher than for peptide 1. In all the cases, the K_m values for the peptides were higher than the K_m for the pure enzyme. All these results suggest that Arg-30 is the most preferred site, but that in the peptides all arginine residues undergo ADP-ribosylation. These results indicate that ADP-ribosylation involves preferentially the two most C-terminal arginines (equivalent positions at Arg-29 and

Figure 3 Effect of ADP-ribosylation on the phosphorylation of PFK-2/FBPase-2

(a) The ADP-ribosylation of PFK-2/FBPase-2 suppressed its phosphorylation by the catalytic subunit of PKA. PFK-2/FBPase-2 was ADP-ribosylated with non-radioactive NAD⁺. After 30 min, the reaction mixture was incubated with [γ -³²P]ATP and catalytic subunit of PKA (lane 3). For controls the same conditions were used, but in the absence of ADPRT (lane 2) or PFK-2/FBPase-2 (lane 1). (b) The phosphorylation of PFK-2/FBPase-2 did not suppress its ADP-ribosylation by ADPRT. PFK-2/FBPase-2 was phosphorylated as described below, but with non-radioactive ATP and, after phosphorylation, the reaction mixture was incubated with [³²P]NAD⁺ and ADPRT (lane 6). For controls the same conditions were used, but in the absence of catalytic subunit of PKA (lane 5) or PFK-2/FBPase-2 (lane 4).

Table 3 Effect of ADP-ribosylation on PFK-2/FBPase-2 activities

PFK-2/FBPase-2 activities were measured after the purified enzyme had been phosphorylated, ADP-ribosylated, or phosphorylated and ADP-ribosylated, and compared with the activities of unmodified enzyme. For more details see the Experimental section. Data are means $\pm$ S.E.M. from three different experiments.

Conditions	PFK-2/FBPase-2 activities (m-units/mg)	
	PFK-2 activity	FBPase-2 activity
Control	31.3 $\pm$ 3.2	3.5 $\pm$ 0.2
PKA	6.6 $\pm$ 0.5	9.2 $\pm$ 3.4
ADPRT	8.3 $\pm$ 0.2	3.7 $\pm$ 0.3
PKA + ADPRT	6.3 $\pm$ 0.6	5.7 $\pm$ 0.8

Arg-30 in PFK-2/FBPase-2), which agrees with the results obtained with the native enzyme.

Effect of ADP-ribosylation on PFK-2/FBPase-2 properties

In order to determine whether ADP-ribosylation of PFK-2/FBPase-2 caused any change in the properties of the bifunctional enzyme, we studied the effect of ADP-ribosylation on the phosphorylation of the enzyme. ADP-ribosylation of PFK-2/FBPase-2 blocked its phosphorylation, although phosphorylation did not suppress ADP-ribosylation (Figure 3).

Since phosphorylation of hepatic PFK-2/FBPase-2 modulates its activity [1–3], we analysed the effect of ADP-ribosylation on the activity of the bifunctional enzyme. Kinase activity decreased when the enzyme was ADP-ribosylated, leading to an activity similar to that of the phosphorylated enzyme, whereas the bisphosphatase was not affected (Table 3).

Table 4 Effect of ADP-ribosylation on the recognition of PFK-2/FBPase-2 by different antibodies

Western-blot analysis of different forms of enzyme using the common or the liver-specific anti-PFK-2/FBPase-2 antibodies. After phosphorylation, ADP-ribosylation, or phosphorylation and ADP-ribosylation, of the enzyme as indicated in the Experimental section, the samples were fractionated by SDS/PAGE (10%), transferred to a membrane and incubated with common or liver-specific anti-PFK-2/FBPase-2 antibodies. The intensity of the autoradiographic signal was quantified by laser densitometry and is presented as a percentage of the signal obtained with unmodified enzyme (control). Data are means $\pm$ S.E.M. from 3–6 different experiments.

PFK-2/FBPase-2	PKA	ADPRT	Common	Specific
+	—	—	100 $\pm$ 8	100 $\pm$ 17
+	+	—	117 $\pm$ 17	103 $\pm$ 11
+	+	+	95 $\pm$ 17	55 $\pm$ 13
+	—	+	94 $\pm$ 19	53 $\pm$ 10

We also determined whether ADP-ribosylation of PFK-2/FBPase-2 modified the recognition of the enzyme by anti-PFK-2/FBPase-2 antibodies. We used two antibodies. One antibody was against liver purified PFK-2/FBPase-2, which we refer to as 'common antibody', since it recognizes different PFK-2/FBPase-2 isoenzymes [28], and another was against a synthetic peptide specific for the N-terminus of hepatic PFK-2/FBPase-2 [31], designated 'liver-specific antibody'. The common antibody recognized PFK-2/FBPase-2 with the same sensitivity whether or not the enzyme was phosphorylated and ADP-ribosylated. However, when the enzyme was ADP-ribosylated, or phosphorylated and ADP-ribosylated, it was poorly recognized by the specific antibody (Table 4).

DISCUSSION

The results presented here provide the first evidence that PFK-2/FBPase-2 can be ADP-ribosylated *in vitro*, with concomitant changes in its immunological and kinetic properties and in its phosphorylation by PKA. PFK-2/FBPase-2 is one of the best known protein substrates for PKA [1,33], and it has been suggested that the presence of three, rather than two, arginines at the phosphorylation site sequence make PFK-2/FBPase-2 an even better substrate *in vitro* for PKA than L-pyruvate kinase or fructose-1,6-bisphosphatase [33]. A similar mechanism may apply to the ADP-ribosylation of these enzymes *in vitro* by ADPRT. The results show that all three arginine residues of the parent synthetic peptide could be ADP-ribosylated, but this modification occurs preferentially at Arg-29 and Arg-30 in the native enzyme. The high affinity of ADPRT for the bifunctional enzyme (0.4 μ M) with respect to the synthetic parent peptide (45 μ M) suggests that the arginines in the native protein are much more readily ADP-ribosylated than those on the peptide. The same argument applies to pyruvate kinase, whose K_m for PKA is lower than for Kempide. It is surprising that the native proteins have higher affinities for ADPRT than do the corresponding PKA phosphorylation-site peptides. The results suggest a requirement for a higher-order structure for ADP-ribosylation. Furthermore, for pyruvate kinase and the bifunctional enzyme the K_m values for ADP-ribosylation are 25-fold and 4-fold lower, respectively, than the K_m values for PKA phosphorylation. Phosphate enhances the rate of ADP-ribosylation at physiological pH, suggesting an enzyme conformational change that favours the covalent modification. These results suggest that the native proteins may be substrates for ADPRT *in vivo*. This is particularly

true for the bifunctional enzyme, with its K_m for ADP-ribosylation which is in the range of the estimated concentration of the protein in liver cytosol [33], whereas its K_m for PKA is 25-fold higher. Since ADP-ribosylation blocks phosphorylation, the relative affinities of the bifunctional enzyme for PKA and ADPRT suggest that ADP-ribosylation could affect the extent of phosphorylation of the bifunctional enzyme *in vivo*. Additional experiments are necessary to establish whether this modification of the enzyme occurs *in vivo*.

ADP-ribosylation was independent of the phosphorylation state of PFK-2/FBPase-2 and suppressed its phosphorylation by PKA. The mechanism by which ADP-ribosylation blocks phosphorylation of PFK-2/FBPase-2 may be similar to that for L-type pyruvate kinase [10], phosphorylase kinase [11] and histone H1 [12], but is unknown. The size of ADP-ribose and the proximity of the phosphorylated serine suggest a steric impediment. Pearson et al. [34] reported that the chemical modification of arginine residues in myosin light chain led to an inhibition of phosphorylation by PKA. Steric interference is also supported by the fact that the ADP-ribosylation of different protein factors with intrinsic activity for ATP or GTP hydrolysis seems to lead to suppression of the activity [35,36].

Neutralization of the positively charged N-terminal arginine residues by negatively charged ADP-ribose may lead to a conformational change in the enzyme molecule that modifies its immunological and kinetic properties. ADP-ribosylation and PKA-catalysed phosphorylation decreased PFK-2 activity to a similar extent, but bisphosphatase activity was not affected by ADP-ribosylation. It has been shown that N- and C-terminal subunit interactions determine the activation state of FBPase-2. Phosphorylation affects the interaction between the two domains and mimics the effects of N- or C-terminal deletions to convert the FBPase-2 domain from a low- to a high-activity-state conformation, although the phosphorylation effect is not as dramatic as deletion of either terminal region. However, uncoupling of the N- and C-terminal interactions influences, but is not necessary for, phosphorylation-induced PFK-2 inactivation [5]. The inactivation of PFK-2 by phosphorylation is probably due to the electrostatic influence of the introduced phosphoryl group, which may interfere directly with fructose 6-phosphate binding and/or induce kinase-domain conformational changes [4,6]. Since ADP-ribosylation does not affect bisphosphatase activity, it seems reasonable to postulate that modification of the arginine residues in the phosphorylation site does not uncouple the N- and C-terminal interaction, as appears to be the case with deletion of either terminal region or PKA-catalysed phosphorylation [6], but acts electrostatically and/or sterically to influence only the PFK-2 active site. Final resolution of the role of the N-terminal region of the enzyme will require elucidation of the three-dimensional structure of the native bifunctional enzyme and both the ADP-ribosylated and phosphorylated forms of the enzyme. The above considerations support the idea that PFK-2 activity and/or net Fru-2,6- P_2 production may be regulated *in vivo* by a balance between phosphorylation and ADP-ribosylation. Experiments are in progress to ascertain whether the enzyme is ADP-ribosylated *in vivo*, but, even if this modification is not physiologically relevant, it provides another probe for studying the role of the N-terminus in controlling the enzyme's activities.

We thank Dr. J. Moss and Dr. L. Hue for generously giving us erythrocyte ADPRT and liver-specific antibody, respectively. We thank also Dr. S. Ambrosio and M. Dalmau for their help and for many valuable suggestions during the course of this work. The skilful technical assistance of C. Ortuño is also acknowledged. X.P. is the recipient of a research fellowship from Fundació Pi i Sunyer (Campus de Bellvitge), and F.V. is the recipient of a research fellowship from the Ministry of Education from

Spain (F.P.I.). This work has been supported by DGICYT (PB91-239) and DGICYT (PM92-248).

REFERENCES

- 1 Pilakis, S. J. and El-Maghrabi, M. R. (1988) *Annu. Rev. Biochem.* **57**, 755–783
- 2 Van Schaftingen, E. (1987) *Adv. Enzymol.* **59**, 315–395
- 3 Hue, L. and Rider, M. H. (1987) *Biochem. J.* **245**, 313–324
- 4 Lin, K., Kurland, I., Li, L. J., Lee, Y. H., Okar, D., Marecek, J. F. and Pilakis, S. J. (1994) *J. Biol. Chem.* **269**, 16953–16960
- 5 Kurland, I. J. and Pilakis, S. J. (1995) *Protein Science*, in the press
- 6 Kurland, I. J., Li, L., Lange, A. J., Correia, J. J., El-Maghrabi, M. R. and Pilakis, S. J. (1993) *J. Biol. Chem.* **268**, 14056–14064
- 7 Ueda, K. and Hayaishi, O. (1985) *Annu. Rev. Biochem.* **54**, 73–100
- 8 Moss, J. and Vaughan, M. (1988) *Adv. Enzymol.* **61**, 303–379
- 9 Mishima, K., Terashima, M., Obara, S., Yamada, K. and Shimoyama, M. (1991) *J. Biochem. (Tokyo)* **110**, 388–394
- 10 Matsuura, R., Tanigawa, Y., Tsuchiya, M., Mishima, K., Yoshimura, Y. and Shimoyama, M. (1988) *Biochim. Biophys. Acta* **969**, 57–65
- 11 Tsuchiya, M., Tanigawa, Y., Ushiroyama, T., Matsuura, R. and Shimoyama, M. (1985) *Eur. J. Biochem.* **147**, 33–40
- 12 Ushiroyama, T., Tanigawa, Y., Tsuchiya, M., Matsuura, R., Ueki, M., Sugimoto, O. and Shimoyama, M. (1985) *Eur. J. Biochem.* **151**, 173–177
- 13 Moss, J., Stanley, S. J. and Watkins, P. A. (1980) *J. Biol. Chem.* **255**, 5838–5840
- 14 El-Maghrabi, M. R., Pate, T. M. and Pilakis, S. J. (1984) *J. Biol. Chem.* **259**, 13096–13103
- 15 El-Maghrabi, M. R., Pilakis, J., Marker, A., Colosia, A., D'Angelo, G., Fraser, B. and Pilakis, S. J. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8430–8434
- 16 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 17 Harlow, E. and Lane, D. (1988) *Antibodies: a Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- 18 Witt, J. T. and Roskoski, R. (1975) *Anal. Biochem.* **66**, 253–260
- 19 Bidlingmeyer, B. A., Cohen, S. A. and Tarvin, T. L. (1984) *J. Chromatogr.* **336**, 93–104
- 20 Glass, D. B. and Smith, S. B. (1983) *J. Biol. Chem.* **258**, 14797–14803
- 21 Glass, D. B. (1983) *Methods Enzymol.* **99**, 119–139
- 22 Kaiser, E., Colescott, R. L., Bossinger, C. D. and Cook, P. I. (1970) *Anal. Biochem.* **34**, 595–598
- 23 Feinberg, R. S. and Merrifield, R. B. (1975) *J. Am. Chem. Soc.* **97**, 3485–3496
- 24 Stewart, J. M. and Young, J. D. (1984) *Solid Phase Peptide Synthesis*, 2nd edn., p. 120, Pierce Chemical Co., Rockford, IL
- 25 Heinrikson, R. L. and Meredith, S. C. (1984) *Anal. Biochem.* **136**, 65–74
- 26 Bartrons, R., Hue, L., Van Schaftingen, E. and Hers, H. G. (1983) *Biochem. J.* **214**, 829–837
- 27 Pilakis, S. J., El-Maghrabi, M. R., McGrane, M., Pilakis, J., Fox, E. and Claus, T. H. (1982) *Mol. Cell. Endocrinol.* **25**, 245–266
- 28 El-Maghrabi, M. R., Correia, J. J., Heil, P. J., Pate, T. M., Cobb, C. and Pilakis, S. J. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 5005–5009
- 29 Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- 30 Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203
- 31 Crepin, K. M., Darville, M. I., Michel, A., Hue, L. and Rousseau, G. G. (1989) *Biochem. J.* **264**, 151–160
- 32 Pilakis, S. J. and Claus, T. H. (1995) *Annu. Rev. Biochem.*, in the press
- 33 Murray, K. J., El-Maghrabi, M. R., Kountz, P. D., Lukas, T. H., Soderling, T. R. and Pilakis, S. J. (1984) *J. Biol. Chem.* **259**, 7673–7681
- 34 Pearson, R. B., Forrest, S., Davis, M., Martin, T. J. and Kemp, N. E. (1984) *Biochim. Biophys. Acta* **786**, 261–266
- 35 Van Dop, C., Yamanaka, G., Steinberg, F., Sekura, R. D., Manclark, R., Stryer, L. and Bourne, H. R. (1984) *J. Biol. Chem.* **259**, 23–26
- 36 Tsai, S. C., Adamik, R., Moss, J., Vaughan, M., Manne, V. and Kung, H. F. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 8310–8314

Received 12 December 1994/31 January 1995; accepted 16 February 1995