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ENDOGENOUS PHOSPHORYLATION OF SOLUBLE ENZYMES IN HUMAN RED CELLS

CYCLIC 3′,5′-AMP-DEPENDENT PHOSPHORYLATION OF PHOSPHOFRUCTOKINASE WITHOUT DETECTABLE REGULATORY EFFECT

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Summary

ATP-depleted human red cells have been incubated in a glucose-containing medium with [^{32}P]orthophosphate in the presence and in the absence of cyclic 3′,5′-AMP and dibutyril cyclic 3′,5′-AMP. Spectrin, pyruvate kinase, phosphofructokinase, glucose-6-phosphate dehydrogenase and hemoglobin A_1 have been purified and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Protein-bound radioactivity has been measured from the sodium dodecyl sulfate polyacrylamide gels and the trichloroacetic acid-precipitated proteins.

In the cytosol, the most intense phosphorylation was found for pyruvate kinase whose, in the presence of cyclic AMP, specific radioactivity was comparable to that of the membrane protein and spectrin. In the absence of cyclic nucleotides it was five times less phosphorylated.

Phosphofructokinase was only phosphorylated when the red cells were incubated with cyclic nucleotides; the extent of phosphorylation was four times less than for pyruvate kinase.

Hemoglobin, glucose-6-phosphate dehydrogenase and a contaminant protein copurified with phosphofructokinase were not phosphorylated: the 'background' of the radioactivity found for these proteins was 100 times less than for pyruvate kinase and spectrin, and 20 times less than for phosphofructokinase (+cyclic AMP).

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Introduction

The regulation of the cell metabolism and function through specific phosphorylation of proteins is now a well-established phenomenon [1]. It has been known for some years that, in the red cells, some membrane proteins are phosphorylated by membrane-bound cyclic AMP-dependent or independent protein kinases [2]. It has been recently shown that red cell cytoplasm also contains protein kinases [3,4]. The function of these cytosolic protein kinases, however, was not known and it was not known whether some soluble enzymes could be phosphorylated by them.

In this paper we show that, in addition to pyruvate kinase whose endogenous phosphorylation has recently been proven [5], both types of subunits of erythrocyte phosphofructokinase can also be phosphorylated by endogenous cyclic AMP-dependent protein kinases; the extent of phosphorylation was approximately four times less than for pyruvate kinase. By contrast, other proteins (hemoglobin, glucose-6-phosphate dehydrogenase and an undetermined contaminant protein copurified with phosphofructokinase) were not or very slightly labeled by [^{32}P]phosphate. Kinetics and stability of red cell phosphofructokinase did not seem to be affected by phosphorylation.

Materials and Methods

Materials

The substrates and auxiliary enzymes were from Boehringer-Mannheim (F.R.G.) or Sigma (St. Louis, MO, U.S.A.). Ion-exchanger resins, CNBr-Sepharose 4B and Dextran blue were supplied by Pharmacia (Uppsala, (Sweden). DEAE-cellulose (DE 52) was from Whatman Biochemical Ltd. (Springfield, U.K.). Acrylamide, bisacrylamide and sodium dodecyl sulfate (SDS) came from Eastman Kodak (Rochester, NY, U.S.A.). Enzyme activities were measured in a Gilford (model 252) spectrophotometer. Absorption at 280 nm of the column eluates was measured with a Gilson apparatus (Model Holochrom). SDS-polyacrylamide gradient slab gels were prepared in the laboratory using an 'Uniscil' gradient former (Universal Scientific Limited, London, U.K.), and the electrophoreses were performed in the electrophoresis apparatus furnished by the same firm. Radioactivity was measured in a Nuclear-Chicago scintillation counter (Model Mark 1, Chicago, IL, U.S.A.). The liquid scintillator used was 'Unisolve 1', from Koch-Light Laboratories Ltd. (Colnbrook, Bucks, U.K.). The trichloroacetic acid-precipitated proteins were collected on Millipore filters (type HA, 0.45 μm) (567120 Molsheim, France). The gel scanner (model DCD-16) was a Gelman apparatus (Gelman Instrument Company, Ann Harbor, MI, U.S.A.).

Methods

Phosphorylation and purification of various red cell protein. Blood, anticoagulated with citrate/phosphate/dextrose and stored under sterile conditions for 6 days at 4°C, was obtained from a blood bank. It was washed three times with a 35 mM Tris-HCl buffer (pH 7.4)/130 mM NaCl/5 mM KCl/1.7 mM $MgCl_2$ (buffer A) containing 0.1 mM Na_2HPO_4, the white cells being carefully

removed. Two samples of 40 ml of red cells were mixed with 60 ml of buffer A containing 20 mM glucose, 1 mM adenosine, 2.5 mM Na_2HPO_4 and 5 mCi [^{32}P]orthophosphate (carrier free), which corresponded to a specific radioactivity of 52 cpm/pmol. After 30 min incubation at 37°C with gentle agitation 0.1 mM cyclic 3′,5′-AMP and 0.1 mM dibutyril cyclic 3′,5′-AMP were added to the first sample and incubation was continued for 4 h. Then the red cells were washed three times in a 20 mM phosphate buffer (pH 7)/100 mM NaCl/50 mM KF.

Hemolysis was provoked by adding to the packed red cells 2 vols. of distilled water containing 1 mM diisopropylphosphorofluoridate, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA and 10 mM β-mercaptoethanol, then KF and phosphate buffer (pH 7) were added to a final concentration of 50 mM and 20 mM, respectively. The membranes were harvested by centrifugation (30 min at 105 000 × *g*), then washed in 5 mM sodium phosphate buffer (pH 8.0) until white.

Membrane proteins were solubilized in 2% SDS with β-mercaptoethanol, heated and resolved by gradient polyacrylamide gel electrophoresis (between 5 and 12.5% acrylamide, w/v) according to Laemmli [6]. Densitometric tracings of the stained gels were obtained with the gel scanner equipped with area integrator. The protein concentration of spectrin was calculated from the areas obtained with proteins of known concentrations. The radioactivity of the spectrin complex (bands 1 + 2) was obtained by slicing the stained gels on the 240 and 220 · 10^3 dalton areas, and counted by Cerenkov radiation.

23 g/100 ml $(NH_4)_2SO_4$ were added to the hemolysates in order to precipitate pyruvate kinase [7,8]; this enzyme was further purified by $(NH_4)_2SO_4$ fractionation and Blue dextran-Sepharose 4B chromatography as previously reported [7,8] except that 40 mM KF instead of KCl were added to all the buffers used.

The supernatant of the 23 g $(NH_4)_2SO_4$ precipitate contained about 75% of the phosphofructokinase and glucose-6-phosphate dehydrogenase activities. Both enzymes were precipitated by raising $(NH_4)_2SO_4$ concentration to 35 g/100 ml of initial hemolysate. This precipitate collected by centrifugation, was washed at 4°C in a 50% $(NH_4)_2SO_4$ solution (buffered at pH 7.5 with solid Tris), 1 mM EDTA, 5 mM β-mercaptoethanol, 0.01 mM $NADP^+$, 0.01 mM fructose-1,6-P_2, 40 mM KF, 20 mM sodium phosphate.

After desalting on a Sephadex G-25 column equilibrated with a 20 mM Tris-phosphate buffer (pH 7.5)/10 mM KF/10 mM $(NH_4)_2SO_4$)/0.01 mM fructose-1,6-P_2/0.1 mM EDTA/10 mM dithiothreitol (buffer B), the preparation was applied on a Blue dextran-Sepharose 4B column [8,9] equilibrated with the same buffer. Phosphofructokinase, but not glucose-6-phosphate dehydrogenase, was fixed on the absorbent; the column was washed with buffer B plus 80 mM KCl until all absorbance at 280 nm has disappeared, then phosphofructokinase was eluted by adding 0.05 mM ADP and 1 mM fructose-6-*P* to the buffer.

In other experiments, phosphofructokinase was purified according to the same method, except that DEAE-cellulose batch treatment was used for the first step of the purification: hemolysate was mixed with the ion exchanger equilibrated with buffer B. The resin was then poured on a Büchner funnel,

washed with buffer B until the eluate was colorless. Phosphofructokinase was eluted with buffer B containing 100 mM potassium phosphate (pH 7.) and 200 mM $(NH_4)_2SO_4$.

Glucose-6-phosphate dehydrogenase coprecipitated with phosphofructokinase on $(NH_4)_2SO_4$ fractionation, but was not bound on the Blue dextran-Sepharose 4B column equilibrated with buffer B. It was further purified by DEAE-Sephadex chromatography and CM-Sephadex chromatography with elective elution by $NADP^+$, as reported previously [10].

On time of the specific elution of the enzymes, a part of the preparation was lyophylised and dissociated by SDS; the other part was mixed with 0.2 mg/ml bovine albumin, then precipitated by $(NH_4)_2SO_4$ (to 80% saturation).

Hemoglobin A_1 was purified by DEAE-cellulose (DE 52) chromatography [11]. Radioactivity of the trichloroacetic acid-precipitated proteins was counted in liquid scintillation, using the filter method as described elsewhere [12]. After SDS-polyacrylamide gradient gel electrophoresis (8—20% acrylamide (w/v), using the discontinuous buffer system described by Laemmli [6]), the protein bands stained with Coomassie blue were scanned, sliced (2 mm thick) and the radioactivity determined.

Kinetic and stability studies of phosphofructokinase. The purified phosphofructokinase samples and intermediate enzymes used for the kinetic studies were extensively dialysed against a 50 mM Tris-HCl buffer (pH 8)/5 mM β-mercaptoethanol/1 mM $(NH_4)_2SO_4$/0.01 mM fructose-1,6-P_2. The reaction mixture was a 50 mM glycylglycine buffer (pH 7.3)/1 mM $(NH_4)_2SO_4$/0.5 mg/ml bovine albumin/5 mM dithiothreitol/100 mM KCl/0.5 mM Mg^{2+} ($MgCl_2$ salt)/0.2 mM NADH/0.5 IU/ml aldolase/10 IU/ml triosephosphate isomerase/0.5 IU/ml glycerol-3-phosphate dehydrogenase, containing various concentrations of fructose-6-*P*. The reaction was started by adding $MgATP^{2-}$ (the experiments were carried out at two different ATP/Mg ratios, 1 : 1 and 1 : 3). All the kinetics were studied at 37°C.

The kinetic behavior towards fructose-6-*P* was studied using a concentration range from 0.025 to 4 mM, at constant concentration of 0.5 mM $MgATP^{2-}$. The kinetic behavior towards $MgATP^{2-}$ was determined using a concentration range from 0.01 to 3 mM, at constant concentration of 1 mM fructose-6-*P*.

Activation by AMP was studied at constant concentration of 0.5 mM fructose-6-*P* and 1 mM $MgATP^{2-}$, AMP concentration being varied from 0.002 to 0.2 mM.

Urea stability was appreciated by incubating the enzyme for various times at 37°C in buffer B (see above), containing 2.5 M urea, with and without 0.01 mM fructose-1,6-P_2.

Results

Enzyme purification

Starting with 40 ml of packed red cells we were able to purify to homogeneity about 100 μg of pyruvate kinase (specific activity: 400 IU/mg [5,7,8]) and 100 μg of glucose-6-phosphate dehydrogenase (specific activity: 160 IU/mg [10]). These amounts were large enough to measure radioactivity, to control their purity by SDS-polyacrylamide gel electrophoresis and to study some kinetic parameters.

TABLE I

PURIFICATION OF ERYTHROCYTE PHOSPHOFRUCTOKINASE

I and II: 6-day-old stored blood, incubated with (I) or without (II) cyclic nucleotides. III: 15-day-old stored blood. Omitting the first purification step of DEAE-cellulose chromatography (samples I and II) results in the copurification, with phosphofructokinase, of a contaminant protein with molecular weight of about 62 000 (see Fig. 1). Enzyme activity was measured at 30°C as indicated in Refs. 9 and 13. Protein concentration was determined according to Lowry et al. [34], with bovine albumin as standard.

	Enzyme activity (I.U.)			Specific activity (I.U./mg of proteins)			Purification (-fold)			Yield (%)		
	I	II	III	I	II	III	I	II	III	I	II	III
Hemolysate	65	49	310	0.007	0.007	0.005	1	1	1	100	100	100
DEAE-cellulose chromatography			300			1.5			300			97
$(NH_4)_2SO_4$ fractionation	36	40	290	0.5	0.7	3.5	71	100	700	55	82	94
Blue dextran-Sepharose 4B chromatography, elective elution with ADP + fructose-6-*P*	16	17	190	72	74	125	10 300	10 600	25 000	25	35	61

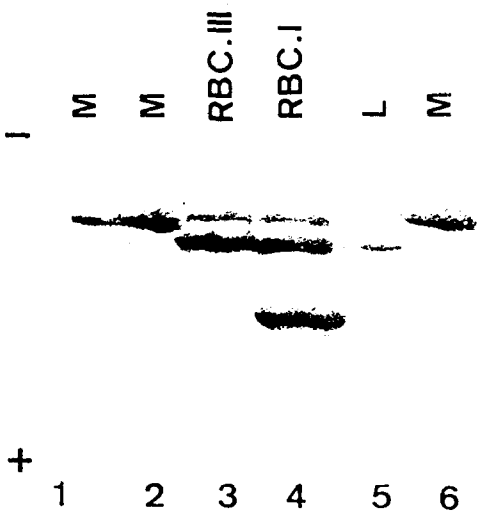

Fig. 1. SDS polyacrylamide gradient gel electrophoresis of different human phosphofructokinase preparations. 5—15 μg of protein were applied per channel. Channel 1, muscle enzyme (preparation a); 2 and 6, muscle enzyme (preparation b); 3, red cell enzyme (preparation III, with DEAE-cellulose chromatography as first step of the purification procedure); 4, red cell enzyme (preparation I); 5, enzyme from chronic myelogenous leukemia leukocytes. Staining with Coomassie blue. M and L refer to the dominant phosphofructokinase subunits of each preparation [9,13]. Muscle enzyme preparations a and b correspond to two different lots of pure M_4 phosphofructokinase.

Total purification of erythrocyte phosphofructokinase requires a first step of DEAE-cellulose chromatography. In these conditions (Table I) we obtained with a high yield (61%) an enzyme preparation with specific activity of 125 IU/mg. This enzyme was composed of two types of subunits with molecular weights of about 85 000 and 81 000 as measured by SDS-polyacrylamide gel electrophoresis [9]. As expected, Fig. 1 shows that the first one probably corresponds to a M-type subunit, whilst the second one corresponds to a L-type subunit [9.13]. The ratio of L/M subunit is approximately 2/1, as already indicated by Karadsheh et al. [14] and Kaur and Layzer [15]. When the first step of DEAE-cellulose chromatography was omitted, as in the case of the purification of phosphofructokinase from the blood samples incubated with [^{32}P]phosphate (preparations I and II, Table I), a third major band was seen on the SDS-polyacrylamide gel; its molecular weight was about 62 000. We do not know the nature of this contaminant protein. The relatively low yield obtained in the purification of enzyme from the incubated samples I and II as compared with preparation III (Table I) probably resulted from the small amount of starting material.

Protein phosphorylation

Table II shows the level of endogenous phosphorylation of various membrane and cytosol proteins, after incubation with [^{32}P]phosphate in the presence or absence of cyclic AMP and dibutyril cyclic AMP. The specific radioactivity of the membrane proteins was 50-fold higher than that of the cytosolic proteins in both conditions. A 2-fold stimulation of phosphorylation of spectrin by cyclic AMP (from $50 \cdot 10^3$ cpm/mg to $92 \cdot 10^3$ cpm/mg) was

TABLE II

PHOSPHORYLATION OF DIFFERENT RED CELL PROTEINS AFTER INCUBATION OF THE WHOLE CELLS WITH [^{32}P]PHOSPHATE, WITH AND WITHOUT CYCLIC NUCLEOTIDES

Hemoglobin, glucose-6-phosphate dehydrogenase and pyruvate kinase were obtained in an homogenous state such that the specific radioactivity was directly measured from the trichloroacetic acid-precipitated proteins. For phosphofructokinase an unlabeled protein was copurified with the labeled enzyme; specific radioactivtiy was calculated by assuming a specific activity of 125 I.U./mg for pure phosphofructokinase. This assumption was validated by the gel scanning shown in Fig. 2: phosphofructokinase accounted for about 60/ of the proteins of these preparations. Spectrin specific radioactivity was estimated from the sliced polyacrylamide gels, as described in Materials and Methods. I, incubation of the red cells with cyclic AMP and dibutyril cyclic AMP. II, incubation without cyclic nucleotides. We have not taken into account the very low radioactivities (less than 200 cpm); these results are noted as 'undeterminable'.

	Enzyme activity		Specific activity		cpm		cpm/mg of proteins		cpm/pmol of subunits	
	I	II	I	II	I	II	I	II	I	II
Total membrane proteins					$3.7 \cdot 10^6$	$2.1 \cdot 10^6$	$125 \cdot 10^3$	$75 \cdot 10^3$		
Purified spectrin							$92 \cdot 10^3$	$51 \cdot 10^3$		
Cytosol										
Glucose-6-*P* dehydrogenase	58	55								
Phosphofructokinase	65	49			$1.85 \cdot 10^7$	$1.44 \cdot 10^7$	$2.4 \cdot 10^3$	$1.8 \cdot 10^3$		
Pyruvate kinase	96	91								
Purified hemoglobin (fraction A_1)							$1.2 \cdot 10^3$	$1.2 \cdot 10^3$	≈0.2	≈0.2
Purified glucose-6-phosphate dehydrogenase	14	13		160	Undeterminable		Undeterminable		Undeterminable	
Purified phosphofructokinase	16	17	72	72	$3.0 \cdot 10^3$	Undeterminable	$23.4 \cdot 10^3$	Undeterminable	1.9	Undeterminable
Purified pyruvate kinase	44.5	38	440	350	$12.6 \cdot 10^3$	$2.8 \cdot 10^3$	$125 \cdot 10^3$	$25.6 \cdot 10^3$	7.9	1.6

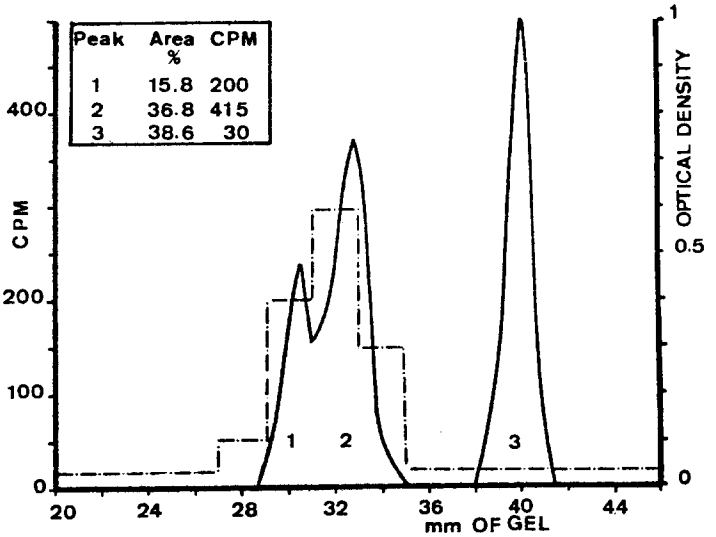

Fig. 2. Scanning of the Coomassie blue-stained polyacrylamide gel (red cell phosphodfructokinase, preparation I) and count for radioactivity. ———, absorbance at 550 nm; · — · — ·, radioactivity per gel slice. Insert represents the relative area of each protein band, and the corresponding radioactivity (Cerenkov radiation). For this experiment each stained band was separately sliced, then counted for radioactivity. Peak I corresponds to the M subunit, peak 2 to the L subunit and peak 3 to the contaminant protein.

observed, similar to the extent of stimulation seen when membrane-bound spectrin was phosphorylated by the endogenous protein kinase. In the cytoplasm, hemoglobin, glucose-6-phosphate dehydrogenase and the contaminant protein copurified with phosphofructokinase (Figs. 1 and 2) were not, or very slightly labeled by [^{32}P]phosphate. By contrast pyruvate kinase was intensively labeled, and its phosphorylation was 5-fold stimulated by cyclic AMP (from 25.6 to 125 · 10^3 cpm/mg).

The pyruvate kinase preparation isolated was composed of about 90% of L′ subunits and 20% of L subunits [5,7]. If we consider a molecular weight of 63 000 for the L′ subunits, this phosphorylation corresponded to about 1.6 and 7.9 cpm/pmol of L′ subunit, respectively, for an initial specific radioactivity of 52 cpm/pmol for extracellular [^{32}P]phosphate. Specific radioactivity of [γ-^{32}P]ATP formed during the incubation was not determined in these experiments.

Phosphofructokinase was also phosphorylated by a cyclic AMP-dependent system, but to a lesser extent than pyruvate kinase: indeterminable without cyclic nucleotide, its specific radioactivity reached 24 · 10^3 cpm/mg in the presence of cyclic AMP, which corresponded to about 2 cpm/pmol of subunits. This was calculated assuming a mean molecular weight of 82 000. As a matter of fact this enzyme is composed of L subunits (of about 80 000 daltons) and M subunits (of about 85 000 daltons), in the ratio 2 : 1 or 3 : 1 according to the preparations (Fig. 1) [9]. Fig. 2 shows clearly that both types of subunits were indistinctely labeled, proportionally to the surface of the protein peak. On the contrary, as indicated above, the 62 000 dalton contaminant protein was not labeled at all.

TABLE III

KINETICS AND STABILITY OF PHOSPHORYLATED AND NOT PHOSPHORYLATED PURIFIED ERYTHROCYTE PHOSPHOFRUCTOKINASE

	ATP/Mg^{2+}	$K_{0.5}$ for fructose-6-*P* (mM)	Hill coefficient	Michaelis constant for ATP (mM)	ATP inhibition (% of inhibition at 1 mM ATP)	AMP activation (μM AMP for a 50% activation)	Urea stability (% of inactivation after 15 min incubation	
							With fructose-1,6-P_2	Without fructose-1,6-P_2
Phosphorylated enzyme	1	0.59	2.7	0.022	73	4	23	36
	1:3	0.64	2.2	0.021	50			
Not phosphorylated enzyme	1	0.65	2.4	0.021	64	5	27	31
	1:3	0.58	2.7	0.026	46			

Kinetics and stability of phosphorylated and not phosphorylated phosphofructokinase

Table III shows that no difference of kinetics or stability between phosphorylated and not phosphorylated enzyme was found: for both enzyme preparations (studied at pH 7.3 and 37°C) $K_{0.5}$ for fructose-6-*P* (at 0.5 mM $MgATP^{2-}$) was from 0.58 to 0.64 mM, Hill coefficient from 2.2 to 2.7; at constant 1 mM fructose-6-*P* concentration, $MgATP^{2-}$ Michaelis-Menten's constant was from 0.021 to 0.026 mM and $MgATP^{2-}$ inhibition was 64—73% of inhibition at 1 mM $MgATP^{2-}$ for a ratio $ATP/Mg^{2+} = 1 : 1$ and 46—50% for a ratio 1 : 3. Half-activation by AMP (at 1 mM $MgATP^{2-}$ and 0.5 mM fructose-6-*P*) was obtained for 4—5 μM AMP. Stability at 37°C in 2.5 M urea was similar for both samples, in the presence as well as in the absence of fructose-1,6-P_2.

Discussion

While phosphorylation of red cell membrane proteins is now a well-documented phenomenon, endogenous phosphorylation of soluble erythrocyte enzymes is reported for the first time. For both phosphorylated enzymes (pyruvate kinase and phosphofructokinase) a cyclic AMP-dependent phenomenon was involved. The protein kinases responsible for this phosphorylation remain unknown. Recently it has been reported that a cyclic AMP-dependent protein kinase type II exists in the red cell cytosol, the cyclic AMP-stimulated membrane enzyme being of type I; both enzymes have the same type of catalytic subunits [4]. Thus it could be that membrane proteins and soluble enzymes are phosphorylated by the two systems, each of them being distinctively regulated.

Some evidence has been recently provided that phosphorylation of spectrin could play a major role in the physicochemical properties of red cell membranes [16] and in the induction of shape change (echinocytes to disc form) [17]. By contrast the hypothetical function of phosphorylation of cytosolic enzymes is more difficult to understand. In the liver, indeed, glucagon-induced phosphorylation of cytosolic enzymes results in the inhibition of glycolysis, stimulation of gluconeogenesis, inhibition of glycogen synthesis and activation of glycogenolysis [18,19]. Except glycolysis, these metabolic pathways do not exist in the red cells. In addition, human red cell adenylate cyclase is not stimulated by hormones [20]. Thus, though phosphorylation of red cell pyruvate kinase is associated with the same type of enzyme inactivation as for liver L-type enzyme (Marie, J. et al., unpublished results), further studies are needed to determine whether endogenous phosphorylation of soluble red cell enzymes plays physiologically a regulatory role.

Our results concerning phosphorylation of erythrocyte pyruvate kinase are in striking disagreement with some recent data by Dahlqvist-Edberg [21], but are easily understood in light of the demonstrated nature of the erythrocyte L′ subunits as precursors of the liver L-type subunits [7,22]. This point and the reasons of the discrepancies with the results of Dahlqvist-Edberg, are discussed elsewhere [5].

Since, in the experiments reported herein, we have not measured specific radioactivity of [γ-^{32}P]ATP during the incubation of the red cells, we cannot

determine the stoichiometry of the radiophosphorus incorporated in the proteins. Nevertheless, it should be noted that the cyclic AMP-stimulated labeling of pyruvate kinase is significantly more than phosphofructokinase (four times more). Recently working in our laboratory with partially purified red cell extracts, we have determined that maximum cyclic AMP-stimulated phosphorylation from [γ-^{32}P]ATP was 3.4 phosphate molecules per pyruvate kinase tetramer and only 0.8 per phosphofructokinase tetramer. These reactions were catalyzed by soluble red cell protein kinases (Marie, J. et al., unpublished results). These results are coherent with data established by other authors, namely that four phosphate molecules are rapidly incorporated per tetrameric liver L-type pyruvate kinase molecule [23] while phosphorylation of phosphofructokinase was shown to be more difficult: Brand and Söling found that, in vitro, incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP in liver enzyme was a rather slow phenomenon [24]. In vivo, muscle enzyme was found to contain a maximum of 1—2 covalently bound phosphate per tetramer [25—27]. The phosphorylation of both liver and muscle phosphofructokinase was reported to be cyclic AMP-independent [24,28]. More recently it has been shown that, in vitro, cytosolic liver cyclic AMP-dependent protein kinase was capable to phosphorylate muscle enzyme and incorporate up to four phosphates per tetramer [29]. In addition pure kidney enzyme could be phosphorylated by the catalytic subunit of kidney cytosolic cyclic AMP-dependent protein kinase [30]. We confirm in this paper that the M-type, as well as the L-type subunits, can, indeed, be phosphorylated by a cyclic AMP-dependent system.

The problem of the specificity of phosphofructokinase phosphorylation is raised in the present work as well as in the above-mentioned papers. This does not signify that we suspect this enzyme to be unspecificially labeled by inorganic phosphate, or on other residues than serine or threonine. We have, indeed, shown that the extent of phosphorylation of phosphofructokinase was 20-fold higher than for the 'unlabeled' proteins (e.g. hemoglobin and glucose-6-phosphate dehydrogenase), and was only detectable in the presence of cyclic nucleotides; phosphorylation from [γ-^{32}P]ATP in partially purified preparations is possible, and yields the same results as endogenous phosphorylation in non-disrupted red cells. In addition, we have recently found that radioactive phosphate could be removed from the enzyme molecules by a 12 h incubation at 37°C in 0.3 M NaOH, which argues for the formation of phosphorylserine residues [31].

The problem is as to whether phosphofructokinase is a sufficiently good substrate of red cell protein kinase(s) for being really phosphorylated in vivo. This can only be determined using partially purified preparations and [γ-^{32}P]-ATP and not whole red cells incubated with exogenous inorganic [^{32}P]phosphate (in these latter conditions, indeed, a long incubation time is indispensable for labeled phosphate entering the cells and labeled ATP being synthesized). It was determined that at high (but physiological) ATP concentration (1 mM), maximum phosphate incorporation was reached in about 5 min. By comparison, pyruvate kinase was fully phosphorylated in about 1—2 min at lower ATP concentration (0.1 mM) (Marie, J. et al., unpublished results). Thus, although it is clear that phosphofructokinase is not as a good substrate for red cell protein

kinase(s) as pyruvate kinase, it seems that phosphorylation of this enzyme is possible in the in vivo conditions. However, as already discussed for pyruvate kinase, we have no evidence that this is really the case.

Brand and Söling are the solely to claim that the cyclic AMP-independent phosphorylation of liver enzyme elicited its activation by stabilizing the tetrameric structure [24]. All the other groups working with muscle and kidney phosphofructokinase were unable to confirm these data. It is clear from our results that the phosphorylation of the erythrocyte enzyme does not modify either kinetic parameters or its stability. Since the phosphofructokinase from erythrocytes incubated without cyclic nucleotide could be incompletely dephosphorylated, we have checked for the properties of the enzyme purified from a crude hemolysate incubated for 2 h at 37°C without phosphate or fluoride, in the presence of 20 mM $MgCl_2$ (to activate protein phosphatases [24]), followed by alkaline phosphatase treatment: this enzyme, expected to be maximally dephosphorylated, exhibited similar specific activity and stability than the phosphorylated phosphofructokinase (data not shown).

Since Brand and Söling reported dephosphorylated liver phosphofructokinase to be stabilized by fructose-1,6-P_2 [24], we tested the influence of this ligand on stability of the enzymes: even in its absence, dephosphorylated red cell phosphofructokinase was as stable as the phosphorylated enzyme. It should be pointed out that activation of liver phosphofructokinase through phosphorylation would be a striking finding since, in the liver, phosphorylation of enzymes of glycogen synthesis, glycogenolysis and glycolysis results, on the contrary, in synthesis of glucose, in particular by inhibiting glycolysis and stimulating gluconeogenesis.

Recently, two groups reported new data suggesting that rat liver phosphofructokinase could be inactivated by cyclic AMP-dependent phosphorylation. Glucagon would be able to stimulate this phosphorylation [32,33]. Such a mechanism, if it is confirmed in further studies, should be more easily understandable in view of the concerted regulation of glycolytic and gluconeogenic pathways in the liver. Our results on red cell enzyme are not inconsistent with these new data: we have shown that, in red cells, phosphofructokinase seemed to incorporate a maximum of one phosphate per tetramer. It could be that in other tissues, such as the liver, the extent of phosphorylation is higher, then resulting in kinetic changes of the enzyme.

In conclusion this paper demonstrates the phosphorylation of some cytosolic enzymes from human red cells by endogenous cyclic AMP-dependent protein kinase. The most intensively phosphorylated enzyme is pyruvate kinase, its specific radioactivity being similar to that of some membrane proteins. Phosphofructokinase is 4-fold less phosphorylated than pyruvate kinase, but its labeling remains highly significant since it is about 20-fold higher than that of 'unphosphorylated proteins' such as hemoglobin or glucose-6-phosphate dehydrogenase. The properties of phosphofructokinase are unchanged by phosphorylation of both M and L-type subunits. Further works are needed to determine whether this phenomenon is physiologically efficient, and whether it plays any regulatory function.

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