Journal of Chromatography, 584 (1992) 77–84
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6596

Purification of glycogen phosphorylase isozymes by metal-affinity chromatography

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ABSTRACT

Mammalian phosphorylase isozymes from muscle, brain and liver were expressed in *Escherichia coli* and purified from the crude bacterial cell extracts in one step using a copper-loaded, metal-affinity matrix. Good chromatographic behavior, enzyme activity and protein stability were maintained by judicious choice of pH and buffer which contained 250 m*M* sodium chloride and 25 m*M* β-glycerophosphate at pH 7.0. Small amounts of β-mercaptoethanol and EDTA in the buffers further stabilized the enzymes, but stripped some of the metal from the column which, nonetheless, retained good chromatographic characteristics. Owing to the presence of multiple surface histidine residues in the phosphorylase dimers, good enzyme purities (90–98%) and recoveries (>90%) were routinely obtained from crude bacterial lysates after two passes through the copper column. Of the various metal ions which were investigated, Cu^{2+} gave the best chromatographic results. Imidazole gradients at constant pH were used to selectively desorb the phosphorylase from the metal column whose capacity for phosphorylase binding in the presence of bacterial proteins exceeded 30 mg enzyme per milliliter of matrix.

INTRODUCTION

Glycogen phosphorylase is a complex allosteric enzyme which catalyzes the phosphorolysis of the α-1,4-glycoside linkage to release glucose-1-phosphate. Phosphorylase plays an essential role in providing chemical energy in virtually all cells and organisms. Rabbit muscle phosphorylase, in particular, has long been investigated as a model for understanding allosteric regulation [1]. Phosphorylase enzymes from other tissues and organisms have been isolated, and their enzymatic activities and allosteric properties have been studied [2]. Unlike muscle tissue, the amount of phosphorylase protein in other tissues or organisms is often only a very small fraction of the total protein and, therefore, more difficult to purify by conventional chromatographic procedures. A congenital deficiency in humans called McArdle's disease involves natural variants of muscle phosphorylase which are difficult to characterize biochemically owing to the small amounts of available protein [3,4]. For these reasons, many studies on phosphorylase enzymes require rapid, high-yield and efficient purification protocols.

In attempting to further understand the structural mechanism of allosteric activation of glycogen phosphorylase, it was essential to obtain protein crystals of natural and variant phosphorylase enzymes for X-ray diffraction experiments. In

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0378-4347/92/$05.00

order to simplify this procedure, we developed a purification procedure based on immobilized metal-ion affinity chromatography (IMAC) [5]. We found protein binding to immobilized copper (II) iminodiacetate (Cu-IDA) to be a reliable first step in obtaining high yields and good purities of all three mammalian phosphorylase isozymes from crude cell extracts. In many cases, the enzymes were sufficiently pure after IMAC purification that good quality crystals could be obtained directly.

EXPERIMENTAL

Materials

The Chelating Superose HR 10/2 column, an iminodiacetic acid (IDA) agarose gel, was purchased from Pharmacia-LKB Biotechnology (Uppsala, Sweden). Imidazole, β-glycerophosphate, EDTA, cupric chloride, and rabbit muscle phosphorylase *b* were obtained from Sigma (St. Louis, MO, USA). Spectra/Por 1 dialysis membrane tubing [6000–8000 molecular weight cut off (MWCO)] was purchased from Fisher Scientific (Pittsburgh, PA, USA). The high-performance liquid chromatographic (HPLC) system was purchased from Rainin Instruments (Emeryville, CA, USA). Protein assay reagents were purchased from Pierce (Rockford, IL, USA). The best results were obtained when IMAC buffers were degassed with helium.

Expression of phosphorylase isozymes

The crude phosphorylase samples used in this study were all obtained from *Escherichia coli* expression systems. Muscle phosphorylase was obtained from *E. coli*, strain 25A6 (W3110, *ton*A, *lonΔ*, *qal*E, *htpP*^{ts}), harboring the expression vector pTACTAC [6]. Human liver phosphorylase was obtained from the same bacterial strain containing the expression plasmid pKK233-2 [7]. The human brain phosphorylase isozyme was obtained from a bacterial expression system using the pTACTAC vector as developed for the muscle enzyme [8].

Purification of phosphorylase isozymes by IMAC

Preparation of crude extracts was done at 4°C and the chromatographic steps were performed at room temperature. Frozen bacterial pellets were thawed and resuspended in 1/12th volume of buffer composed of 25 m*M* β-glycerophosphate, 0.3 m*M* fresh β-mercaptoethanol, 0.3 m*M* EDTA, 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin and 0.01% benzamidine · HCl. Bacterial cells were disrupted using a Branson 250 sonifier. The sonicated sample was centrifuged for 45 min at 17 400 *g* to remove insoluble material. The crude supernatant was filtered through a 0.45-μm filter before chromatography.

The clean Chelating Superose column was washed with 100 m*M* NaCl, loaded with 50 m*M* $CuCl_2$, washed again with 100 m*M* NaCl, saturated with the release buffer (100 m*M* imidazole, 250 m*M* NaCl, 25 m*M* β-glycerophosphate, pH 7.0) and finally equilibrated with the starting buffer (1 m*M* imidazole, 250 m*M* NaCl, 25 m*M* β-glycerophosphate, pH 7.0). The soluble, crude cell extract (20 ml) was then applied directly to the copper column which was finally washed with the starting buffer until the absorbance of the effluent reached that of the starting buffer itself. The bound proteins were eluted with a linear 1–100 m*M* imidazole gradient at a flow-rate of 60 ml/h. The protein concentration of column eluates was monitored by measuring the absorbance at 280 nm. Fractions (0.5 ml) were collected in receiving tubes which also contained EDTA and β-mercaptoethanol so that the final concentration for each reagent was 0.5 m*M* after dilution with the eluate. Pooled fractions were dialyzed against 25 m*M* β-glycerophosphate (pH 7.0) which also contained 0.3 m*M* EDTA and 0.3 m*M* β-mercaptoethanol. The protein concentration of purified samples was determined from the absorbance at 596 nm by Pierce Protein Assay Reagent based on the Bradford method [9].

This one-step purification of phosphorylase from crude cell extracts yielded enzyme purities in the range 80–85% [sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), reversed-phase HPLC]. A significant fraction of

the impurity protein resulted from non-specific binding of cellular and membrane proteins to the matrix. The pooled phosphorylase fractions were subjected to buffer exchange and concentration (1–3 m*M* imidazole, 25 m*M* β-glycerophosphate, 250 m*M* NaCl, 0.3 m*M* β-mercaptoethanol, 0.3 m*M* EDTA) before reloading on the same, cleaned IMAC column and elution using the same protocol. The second pass through the Cu-IDA column resulted in phosphorylase purities of 90–98% with excellent recoveries (~95%). The Chelating Superose column was cleaned by removing the copper with 50 m*M* EDTA and then washing with 100 m*M* NaOH solution. The column was then washed with 100 m*M* NaCl, then with 25 m*M* β-glycerophosphate (pH 7.0) and finally with 100 m*M* NaCl in preparation for metal loading.

Final ion-exchange purification of phosphorylase

The IMAC-purified phosphorylase could be further purified by passing the enzyme through DEAE Sepharose Fast-Flow. The phosphorylase eluted early in the salt gradient leaving impurity proteins behind [6,10].

Activity assays

The specific activity of the phosphorylase isozymes was determined by measuring the rate of phosphate released upon the incorporation of glucose-1-phosphate into glycogen [11]. Column fractions were analyzed on denaturing 10% polyacrylamide gels [12] and stained with Coomassie Brilliant Blue G-250. The identity of rabbit muscle phosphorylase was confirmed by immunoblot analyses [6].

RESULTS AND DISCUSSION

Traditional methods of purification of muscle phosphorylase required multiple steps of ion-exchange chromatography or repeated cycles of recrystallization [13]. In this study we show that rabbit muscle, human liver and human brain glycogen phosphorylase isozymes interact specifically with Cu-IDA (Fig. 1A). Thus, in a single purification step, each enzyme was purified to greater than 80% purity from crude cell extracts prepared from bacterial expression systems. The vast majority of bacterial proteins in the crude extract either did not bind or bound relatively weakly to the metal column and were washed off with the equilibration buffer which contained 1 m*M* imidazole. All phosphorylase isozymes were eluted only after the application of an imidazole gradient (1–100 m*M*). Each isozyme eluted at a different concentration of imidazole as indicated by the arrows in Fig. 1A.

Fig. 1B shows the SDS-PAGE analysis of the major peak eluted from the Cu-IDA column during the development of the imidazole gradient (lanes 1–4) for each phosphorylase isozyme. Most of the contaminating *E. coli* proteins present in the crude extract (lane L) were not present in the eluted fractions which contained the phosphorylase isozymes. The identity of muscle phosphorylase was confirmed by immunoblot analyses (data not shown). The identity of all of the isozymes was further verified by measurement of enzymatic activity. Recovery of purified phosphorylase enzymes with high specific activities was dependent on the presence of small amounts of EDTA and β-mercaptoethanol in the loading and storage buffers. Furthermore, the presence of β-glycerophosphate in the buffers significantly improved enzyme stability. The phosphorylase enzymes were stored at 4°C in β-glycerophosphate buffer which contained 0.3–0.5 m*M* EDTA and β-mercaptoethanol both before and after purification. In order to maintain full enzymatic activity during the IMAC purification, EDTA and β-mercaptoethanol were added to each tube of the fraction collector. We found the hydrogen ion concentration to be an important factor; specific activity of rabbit muscle glycogen phosphorylase at pH 6.1 was half that at pH 7.0. The presence of EDTA and β-mercaptoethanol in the loading buffers caused metal to be stripped from the top 10–20% of the IMAC column; however, the remaining metal-loaded column was fully capable of carrying out the purifications. Virtually all of the copper lost from the column was present in the first few fractions, and only trace amounts of metal (1–3 ppm) were found in the

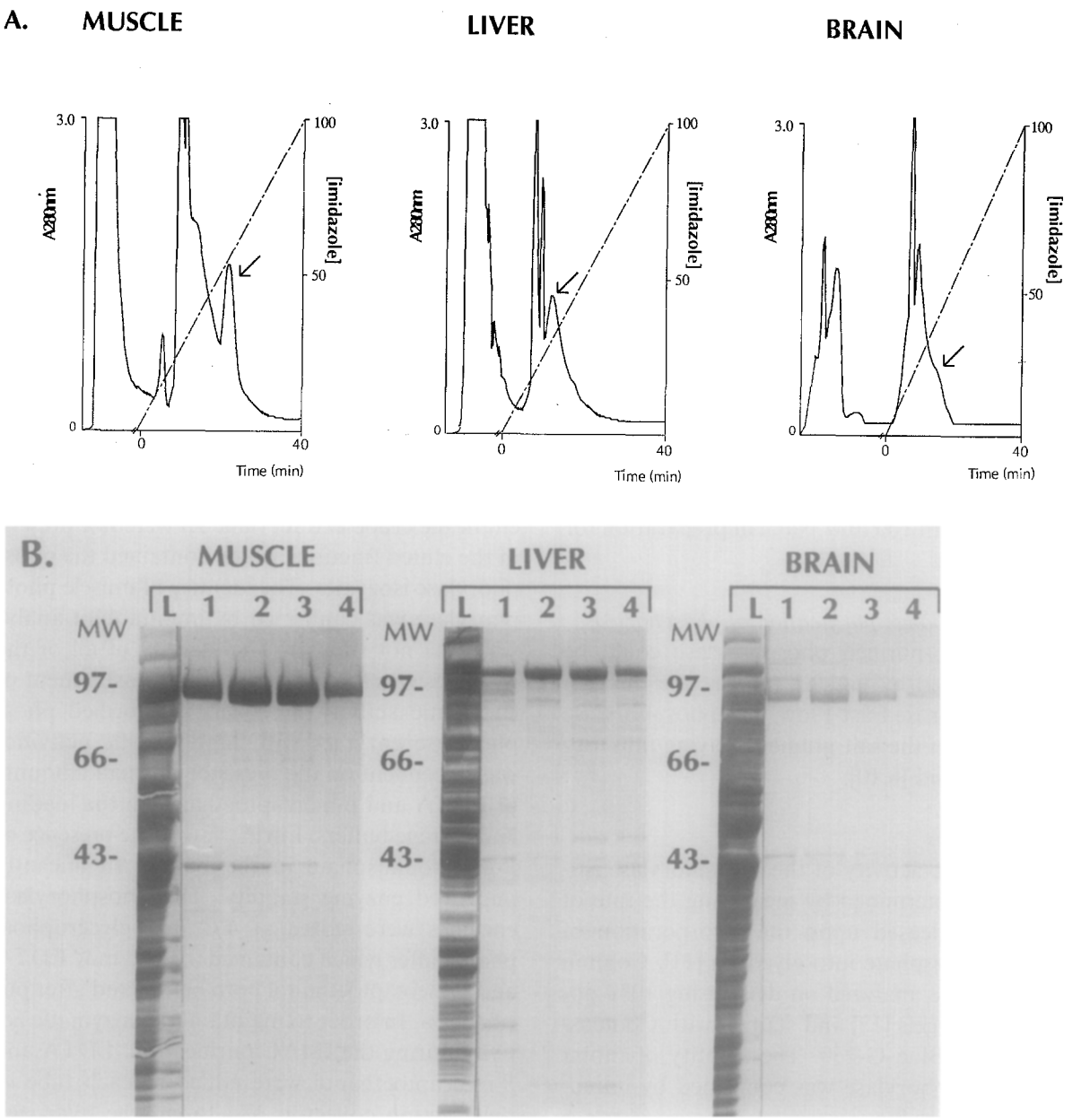

Fig. 1. Purification of glycogen phosphorylase isozymes by IMAC. (A) Absorbance (280 nm) profile of immobilized Cu-IDA chromatography. During the development of a linear imidazole gradient (1–100 m*M*, dashed lines), each phosphorylase isozyme was eluted as shown by the arrows. (B) SDS-PAGE analysis of phosphorylase isozymes after a single IMAC purification. The soluble crude bacterial extract that was loaded on the column is shown (L), along with the peak fractions containing each phosphorylase isozyme (lanes 1–4).

pooled phosphorylase fractions. These trace quantities of fully sequestered metal did not interfere with the action of the enzyme and were removed (< 10 ppb) by dialysis with the storage buffer. X-Ray diffraction studies on crystals of IMAC-purified muscle phosphorylase showed no evidence for the presence of any metal. Depending on the volume and the concentrations of strong metal chelators and thiols in the load mixture, an IMAC column can usually be packed

long enough so that sufficient column length and capacity remain after sacrificing the top of the column during loading.

In the absence of surface-accessible cysteine residues (free –SH), exposed histidine residues are primarily responsible for the binding of various proteins to an immobilized metal affinity matrix. The number of exposed histidine residues and their respective electronic and steric environments are the primary determinants in the chromatographic profile of closely related proteins [14]. In considering the roles of amino acid residues which would be accessible to a ligated metal in phosphorylase isozymes, we determined the number and positions of surface histidine and cysteine residues from the X-ray structure of the rabbit muscle phosphorylase dimer [15]. There were no surface-accessible cysteine residues in the enzymes, and the exposed histidines are listed in Table I. Using published sequence alignments of phosphorylase isozymes [2], the rabbit muscle structure was used to predict the relative accessibility of histidine residues in the other isozymes for which no three-dimensional structures are known. Based on these observations, the order of relative affinity for Cu-IDA would be brain > muscle > liver. The chromatographic results showed, however, that the actual binding order was muscle > brain > liver. The muscle isozyme eluted from the Cu-IDA matrix at 52 m*M* imidazole while the brain and liver isozymes eluted at 43 and 37 m*M* imidazole, respectively. It is clear that neighboring charged (Lys, Arg) or bulky residues can adversely affect metal binding, but these factors are difficult to quantify from the structural models.

The general utility of immobilized metal-ion affinity in the purification of phosphorylases from a variety of sources is supported by the sensitivity and yield of the procedure. Fig. 2A shows a dilution series where crude cell extracts containing muscle phosphorylase were diluted with similar crude cell extracts which contained no expressed phosphorylase protein. As the amount of phosphorylase was reduced from 1.0% (1:1) to 0.02% (1:50) of total protein, the resolution of the phosphorylase peak did not change. Recovery of the muscle phosphorylase also remained unchanged with greater than 90% yield in all cases (Fig. 2B). The good recovery of the phosphorylase isozymes from diluted bacterial extracts suggests that IMAC purifications using copper should also be useful in obtaining phosphorylase from mammalian tissue extracts. As seen in these dilution experiments, there was a minor 43-kDa protein which copurifies with muscle phosphorylase. This impurity was not reactive with the polyclonal antibodies against muscle phosphorylase on immunoblot assays. The 43-kDa protein band was also not observed when phosphorylase eluted earlier in the imidazole gradient (Fig. 1B), providing further evidence that it was not a phosphorylase degradation product, but rather an *E. coli* protein. We have also observed the same or similar 43-kDa protein in Cu-IDA purifications of other proteins expressed in *E. coli*.

Different metal ions were evaluated as to

TABLE I

PREDICTION OF EXPOSED HISTIDINE RESIDUES IN GLYCOGEN PHOSHORYLASE DIMERS

Enzyme[a]	Total number of His residues	Buried	Partly exposed	Exposed	Elution imidazole concentration (m*M*)
Muscle	44	22	6	16	52
Liver	38	24	6	8	37
Brain	52	26	6	20	43

[a] Predicted accessibility to ligated copper, copper (II) N-methyliminodiacetate, without regard to local electronic environments. Liver and brain enzymes were assumed to be isostructural with the muscle enzyme.

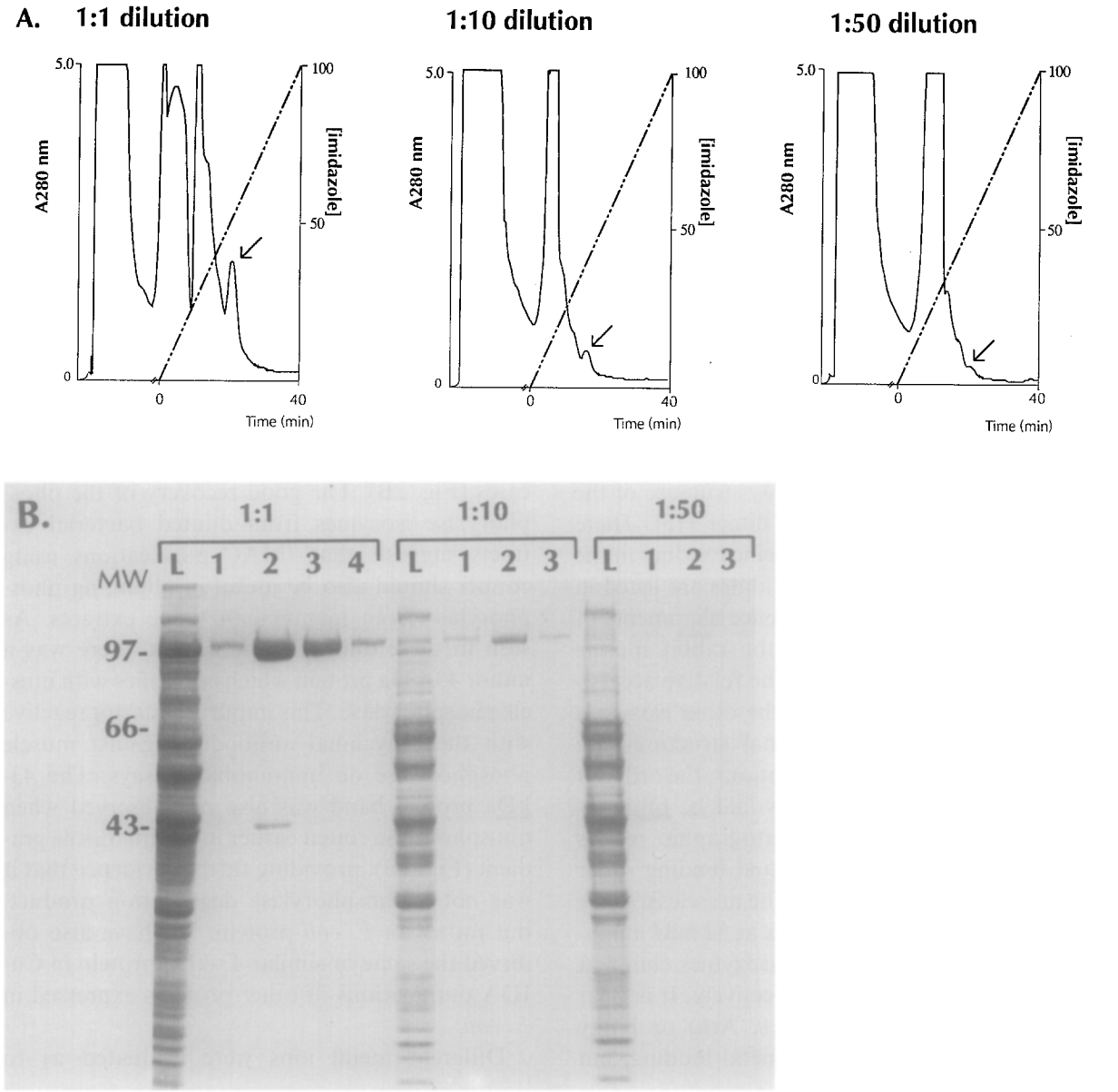

Fig. 2. Dynamic range of IMAC purification. (A) Absorbance profile of IMAC purification of muscle phosphorylase. Muscle phosphorylase from crude bacterial extract containing muscle phosphorylase, serially diluted by the addition of extract without phosphorylase samples (1:1, 1:10, 1:50), was eluted at approximately the same imidazole concentration (dashed line), 52 m*M*, as indicated by the arrows. (B) SDS-PAGE analysis of muscle phosphorylase purification from diluted extracts. The amount of phosphorylase in the starting extract (L) varied from 0.02 to 1.0% of total protein. Greater than 90% of the phosphorylase was always recovered in the peak fractions (lanes 1–4).

which would function best with the Chelating Superose matrix in purifying phosphorylase isozymes. Using our buffer conditions (250 m*M* NaCl, 25 m*M* β-glycerophosphate, pH 7.0) Cu^{2+} and Ni^{2+} gave acceptable to good separations and recoveries, while Zn^{2+}, Co^{2+}, Fe^{2+} and Fe^{3+} gave poor results owing to excessive loss of metal or poor protein binding to the column. In general, Cu-IDA matrices gave the best results. An earlier report has shown that a chicken mus-

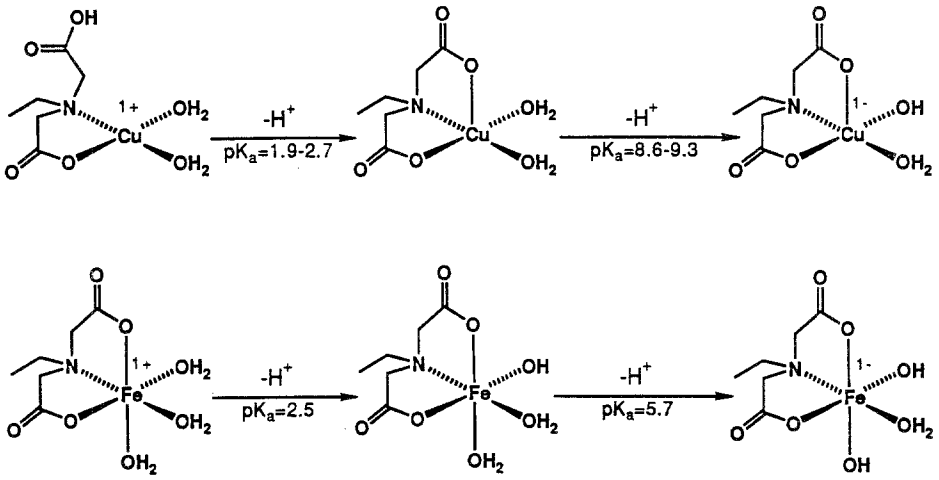

Fig. 3. Forms of IDA–copper and IDA–iron complexes at different pH values.

cle phosphorylase-like enzyme could be purified using Fe(III)-IDA immobilized on Sepharose Fast-Flow with phosphate-free buffers [16]. Using the literature buffer (1 *M* NaCl, 20 m*M* Tris, pH 7.7) or our own buffer (500 m*M* NaCl, 25 m*M* MES, 25 m*M* MOPS, 25 m*M* N,N′-bis(3-sulfopropyl)piperazine (PIPS) [17], pH 8.0) we found that rabbit muscle phosphorylase bound to the Fe(III)-IDA column and could be eluted using either a descending pH gradient (8.0–6.0) or an increasing imidazole gradient (0.2–20 m*M*) at constant pH (7.0). Immobilized (IDA)Fe $(OH_2)_3^{1+}$ undergoes two sequential deprotonations [18] near pH values of 2.5 and 5.7 forming the anionic dihydroxy–iron complex[a], (IDA)Fe $(OH)_2(OH_2)^{1-}$, which acts as a cation exchanger and a weak histidine binder (Fig. 3). In contrast, (IDA)Cu$(OH_2)_2$ exists in this neutral form [19–21] over a wide pH range of 3–8 (Fig. 3). Immobilized Cu-IDA columns have much weaker ion-exchange properties, and they bind exposed histidine residues much more strongly. The copper column is compatible with a wider pH range, lower salt concentrations and the presence of phosphate-containing buffers. In addition, the copper column gives better chromatographic resolution and has significantly higher protein binding capacity. As far as we can ascertain from our results in the presence of salt, rabbit muscle phosphorylase binds to (IDA)Fe$(OH)_2(OH_2)^{1-}$ and to (IDA)Cu$(OH_2)_2$ in the same manner, primarily through histidine coordination to the immobilized metal. Imidazole and histidyl side-chains are known to bind strongly to Cu^{2+}, Fe^{3+} and (IDA)Cu$(OH_2)_2$. However, it appears that the hydroxy ligands in (IDA)Fe$(OH)_2(OH_2)^{1-}$ significantly reduce its binding efficiency toward imidazole-like ligands. This parallels the observations that (IDA)Fe$(OH_2)_3^{1+}$ is an effective catalyst at low pH, but the complex is a poor catalyst at intermediate pH values when iron–hydroxy complexes are readily formed [22].

The convenient IMAC purification of glycogen phosphorylases has greatly facilitated the investigation of the diverse regulatory properties of these complex allosteric enzymes. After two passes through the Cu-IDA column, purified (98%) muscle phosphorylase is readily crystallized. The non-specific binding of certain proteins from the

[a] These values were determined for N-(2-hydroxyethyl)iminodiacetic acid; the corresponding values for iminodiacetic acid itself have not been measured. It should be remembered that immobilized IDA often contains a substituted N-(2-hydroxyethyl) group as part of the linker because epichlorohydrin (glycidyl chloride), a glycidyl ether or other epoxide is frequently used to attach the IDA to the chromatographic matrix. It is possible that the 2-hydroxyethyl group can reach around the metal and occupy a fourth coordination site and that the first or second acid dissociation of such a metal complex could represent loss of H^+ from the ligated alcohol rather than from ligated water.

crude lysate to the IMAC matrix followed by non-specific release appears to be partly responsible for the impurities seen after the first pass through the IMAC column. We have found some variants of rabbit muscle glycogen phosphorylase which would not crystallize when the enzymes were purified only by traditional ion-exchange chromatography methods, but which formed diffraction quality crystals when purified using two cycles of IMAC chromatography. When necessary, the minor impurities remaining in the phosphorylase samples after one or two cycles of IMAC purification can be removed by ion-exchange chromatography.

ACKNOWLEDGEMENTS

This work was partially supported by grants from N.I.H. (DK26081) and Monsanto Company to R.J.F.

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