Characterization of the Zinc-binding Site of the Histidine-Proline-rich Glycoprotein Associated with Rabbit Skeletal Muscle AMP Deaminase*

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The AMP deaminase-associated variant of histidine-proline-rich glycoprotein (HPRG) is isolated from rabbit skeletal muscle by a modification of the protocol previously used for the purification of AMP deaminase. This procedure yields highly pure HPRG suitable for investigation by x-ray absorption spectroscopy of the zinc-binding behavior of the protein. X-ray absorption spectroscopy analysis of a 2:1 zinc-HPRG complex shows that zinc is bound to the protein, most probably in a dinuclear cluster where each Zn²⁺ ion is coordinated, on average, by three histidine ligands and one heavier ligand, likely a sulfur from a cysteine. 11 cysteines of HPRG from different species are totally conserved, suggesting that five disulfide bridges are essential for the proper folding of the protein. At least another cysteine is present at different positions in the histidine-proline-rich domain of HPRG in all species, suggesting that this cysteine is the candidate for zinc ligation in the muscle variant of HPRG. The same conclusion is likely to be true for the six histidines used by the protein as zinc ligands. The presence in muscle HPRG of a specific zinc-binding site permits us to envisage the addition of ligands. This paper is available on line at http://www.jbc.org

mRNA is localized specifically to the liver, suggesting that the previously described HPRG expression by immune cells is due to the acquisition of the plasma protein derived from the liver (2). In a previous paper we reported that denaturation of rabbit skeletal muscle AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) in acidic medium allows the chromatographic separation of the enzyme from a peptide with an amino acid composition significantly different from that derived from the available AMP deaminase cDNAs. N-terminal sequence analysis of the fragments liberated by limited proteolysis revealed a striking similarity of the novel protein to rabbit plasma HPRG although, in comparison with mature HPRG, the AMP deaminase-associated variant probably contains a unique N-terminal extension (3). We now report that the AMP deaminase-associated variant of HPRG can be isolated from rabbit skeletal muscle by a modification of the protocol used in our laboratory for the purification of AMP deaminase. The modification allows the partial dissociation of HPRG at a high degree of purity from the cellulose phosphate-bound enzyme.

Rabbit plasma HPRG contains 5 histidine residues, of which 34 are located in the histidine-proline-rich domain containing 15 repeats of the sequence (H/P)(H/P)PHG that has been proposed to mediate interactions with transition metals, although no evidence of a specific binding has been given (4). The HPRG component of rabbit skeletal muscle AMP deaminase contains 10 mol of histidine residues/10,000 g of protein (3). The abundance of such potential metal ligands suggests that HPRG has the ability and perhaps the function to bind several metal ions, and it has been established (5, 6) that HPRG from rabbit serum binds Hg²⁺, Cu²⁺, Zn²⁺, Ni²⁺, Cd²⁺, and Co²⁺ in descending order of binding affinity. However, no attempts to characterize the structure of the metal-binding site(s) of HPRG have been performed. This prompted us to an investigation by x-ray absorption spectroscopy of the zinc-binding sites of the HPRG variant that we have isolated from rabbit skeletal muscle as a first step toward determining the physicochemical properties of this novel protein.

EXPERIMENTAL PROCEDURES

Reagents—Chelating Fast Flow Sepharose was from Amersham Biosciences. Phosphocellulose resin (P-11) was supplied by Whatman International Ltd., Maidstone, UK. All of the chemicals and other reagents used were of analytical grade.

AMP Deaminase and Its HPRG Component—AMP deaminase was prepared as described previously (7) from fresh muscle dissected from the back and hind leg of rabbits. Because rabbit skeletal muscle AMP deaminase undergoes progressive fragmentation with storage, homogenization of the muscle and phosphocellulose purification of the enzyme was carried out at each step using a buffer system containing 5 mM...
Zinc-binding Site of Skeletal Muscle HPRG

NaCN, to reduce the rate of proteolytic processes. In the presence of azide, the 85–70-kDa bands of the purified enzyme occurred with a half-time of one month, significantly slower than that previously described (half-time of 2 weeks) (8). Following the identification (described in the present paper) of the peptides that elutes at 0.6 M KCl from cellulose phosphate-bound AMP deaminase with the HPRG component of the enzyme, the fractions that formed the peak eluted with 5 mM NaNO₃ (0.6 M KCl, pH 7.0) were concentrated by ultrafiltration and ion exchanged with an Amicon Microcon YM-30 centrifugal filter device (Millipore) and stored in aliquots at −20 °C. An 1 mM HPRG sample was prepared for XAS by further concentration of the pooled fractions. The protein concentrations of the various enzyme fractions were determined spectrophotometrically by using λmax values of 9.1 and 8.2, respectively, for AMP deaminase and its isolated HPRG component, which were calculated on the basis of protein determinations by the method described in Ref. 9 using bovine serum albumin as standard.

The isolated rabbit skeletal muscle variant of HPRG was assumed to have the same molecular mass of 58 kDa calculated for the rabbit plasma protein (4). The AMP deaminase activity was determined spectrophotometrically as previously described using a Shimadzu UV-260 spectrophotometer (10).

SDS/PAGE—Electrophoresis in the presence of 0.1% (w/v) SDS was carried out under reducing conditions on 10% (w/v) polyacrylamide slab gels in 0.1 M Tris, 0.1 M Bicine, pH 8.3. Protein standards (Sigma) were run under reducing conditions on 10% (w/v) polyacrylamide slab gels to determine molecular weights.

Electrophoretic and Sequence Analysis—Electrophoresis was performed by the method of LeGendre and Matsudaira (11) using 10% (w/v) CAPS, pH 11, containing 10% (w/v) methanol. Transfers were performed for 60–90 min at 400 mA. N-terminal sequencing was performed using an Applied Biosystems model 476A protein sequencer.

UV Spectra—The interaction of rabbit muscle HPRG with metals was assessed by changes in absorption. Absorption spectra were recorded by using a Shimadzu UV-260 spectrophotometer at room temperature. Spectral measurements were made within 1 min of mixing protein with metal, and no absorbance changes after this time were noted.

Equal concentrations of metal in the reference cuvette served as reference solutions. The fractional saturation of HPRG, α, is defined as the ratio of the observed increase in absorbance (A) at 275 nm to the maximum AΔ at saturation of the protein.

XAS Sample Preparation—Two equivalents of Zn²⁺ from a ZnSO₄ solution in ultrapure water were added to 1.0 mM rabbit muscle HPRG solution (prepared as described above) to obtain 50 μl of Zn-HPRG 2:1 complex. 45 μl of the above solution were filled into a plastic cell covered with Kapton windows. Both the cells and the Kapton foils used for the sample collection were washed with 0.6 M KCl (peak 1) and with 1.0 M KCl, indicating its correspondence to the peptide previously discarded as a contaminant of the enzyme preparation.

XAS Data Collection and Analysis—The XAS data were collected at Deutsches Elektronen Synchrotron (DESY) (Hamburg, Germany) at the EMBL bending magnet beam line D2 using Si (111) double monochromator for the measurement at the zinc edge. During the measurements the DESY storage ring was operating under normal conditions (4.5 GeV, 90–140 mA). Ionization chambers in front and behind the sample were used to monitor the beam intensity. The XAS data have been recorded by measuring the zinc-Kα fluorescence using a Canberra 13-element solid state detector over the energy range between 9324 and 10624 eV using variable energy step widths. In the x-ray absorption near-edge structure and the extended x-ray absorption fine structure (EXAFS) regions, steps of 0.3 and 0.5–1.2 eV were used, respectively. An indication of the quality of the data obtained from the purified enzyme is given in the figure where multiple scattering contributions were included for histidine imidazole ligands. A zinc-bound imidazole ring was generated by molecular modeling with a zinc-N(His) distance obtained from the EXAFS first shell analysis. The coordinates from the model were input to EXCURVE9.20 to provide a single unit with the correct geometry. A single distance fit was able to reproduce the closer atomic shell, allowing us to minimize the number of parameters in the refinement procedure by simulating the zinc-histidine ligands by a single zinc-bound imidazole ring with a fixed coordination number. In this way the imidazole outer shell distances were defined by the zinc-N(His) distance, and the imidazole outer atoms were constrained to vary with R 0.1 Å from the idealized positions. All of the histidine numbers between 1 and 4 were tried with 3, giving the correct fit. The imidazole ring plane was kept coincident with the zinc-nitrogen bond throughout the refinement.

The quality of the fit obtained was assessed by the following goodness-of-fit function,

\[
\chi^2 = \frac{1}{N_{\text{data}} - p} \sum \left[ \frac{\exp(k_{\Delta}(r)}{k_{\Delta}(r)} - 1 \right]^2
\]

where \(N_{\text{data}}\) is the number of independent data points, \(p\) is the number of parameters, \(N\) is the number of data points, and \(w\) is the weight of the spectrum. The quality of the fit obtained was also assessed by the R-factor as defined within EXCURVE9.20 as follows.

\[
R_{\chi^2} = \frac{\sum \left[ \frac{\exp(k_{\Delta}(r)}{k_{\Delta}(r)} - 1 \right]^2 \times w}{100}
\]

RESULTS AND DISCUSSION

Selective Purification of the AMP Deaminase-associated HPRG by Cellulose Phosphate Ion Exchange Chromatography.—A rapid method for the preparation of AMP deaminase from frozen rabbit skeletal muscle was introduced on the basis of the observation that the enzyme remained bound to cellulose phosphate under conditions (0.45 M KCl, pH 7.0) at which apparently no other proteins are bound (15). Thus, elution with 1.0 M KCl, pH 7.0, yielded a homogeneous preparation of the enzyme at a high degree of purity. However, by eluting cellulose phosphate column with a linear gradient from 0.45 to 1.0 M KCl, sometimes a small protein peak that was not examined was found by the same authors to precede the main activity peak (16). The constant presence of presumably the same additional peptide was observed in the enzyme prepared in our laboratory from fresh rabbit muscle. Therefore, we introduced modification in the cellulose phosphate chromatography (i.e., the enzyme was eluted with 1.0 M KCl after the column had been washed with 0.6 M KCl) that effectively separated the contaminant peptide from the purified enzyme (7). We have now found that the HPRG component of rabbit skeletal muscle AMP deaminase is dissociated from the cellulose phosphate-bound enzyme at a high degree of purity by elution with 0.6 M KCl, indicating its correspondence to the peptide previously discarded as a contaminant of the enzyme preparation.

Fig. 1 shows the elution profile of cellulose phosphate-bound AMP deaminase obtained by two successive washing steps with 0.6 and 1.0 M KCl, after having washed the column with 0.45 M KCl. In five separate enzyme preparations the yield of the AMP deaminase peaks (peak 1) were concentrated by ultrafiltration and the XAS extraction has been performed by fitting a cubic spline using the EXPROG program package (13).

The full \(k^2\) weighted EXAFS spectrum (23–750 eV above \(E_0\)) and its Fourier transform (FT) calculated over the range 3.0–14.0 Å⁻¹ were compared with the theoretical simulations obtained by the set of programs EXCURVE9.20 (14). The edge energy \(E_0\) was adjusted at the beginning of the refinement to bring the experiments and the simulations on the same scale and left unchanged during the fitting. A fixed amplitude factor of 0.95 was used to compensate for amplitude reduction of the signal caused by multiple excitations. The \(k^2\) weighted full spectrum was simulated by varying the atom types and the coordination numbers (as integers) and iteratively refining the distance (r) and the Debye-Waller factor (2σ²) for each atomic shell. Multiple scattering contributions were included for histidine imidazole ligands. A zinc-bound imidazole ring was generated by molecular modeling with a zinc-N(His) distance obtained from the EXAFS first shell analysis. The coordinates from the model were input to EXCURVE9.20 to provide a single unit with the correct geometry. A single distance fit was able to reproduce the closer atomic shell, allowing us to minimize the number of parameters in the refinement procedure by simulating the zinc-histidine ligands by a single zinc-bound imidazole ring with a fixed coordination number. In this way the imidazole outer shell distances were defined by the zinc-N(His) distance, and the imidazole outer atoms were constrained to vary with \(R\) 0.1 Å from the idealized positions. All of the histidine numbers between 1 and 4 were tried with 3, giving the correct fit. The imidazole ring plane was kept coincident with the zinc-nitrogen bond throughout the refinement.
kDa, but an additional faint 95-kDa band that was almost completely proteolyzed in the first week of storage at 4 °C was present in the electrophoretogram of pool 2 (Fig. 2B, lanes 1 and 2).

With aging of pool 2, a 85–70-kDa band transition also occurred with a half-time of 1 month (Fig. 2B, lanes 2–5), confirming our previous demonstration that limited proteolysis of rabbit skeletal muscle AMP deaminase, removing the 95-residue-long N terminus of the enzyme, converts the native 85-kDa subunit to an approximately 70-kDa core that is resistant to further proteolysis (17). In contrast, the 85-kDa band of pool 1 was almost completely transformed in the first week of storage, giving rise to a 95-kDa band that was resistant to proteolysis, even if the enzyme was stored at 4 °C for several months (Fig. 2A, lanes 2–5). By electroblotting and sequencing analysis, the 85-kDa original band of each pool yielded no N-terminal sequences, confirming the previous suggestion that the N terminus of rabbit skeletal muscle AMP deaminase is modified (3).

In contrast, the 95-kDa bands revealed the single sequence LTPTDXXKTTKPL corresponding to the N-terminal sequence of rabbit plasma HPRG. The yield of the sequence increased after storage for few days at 4 °C, indicating that the native protein has a blocked N terminus and that it undergoes a proteolytic process starting with its isolation. It should be noted that rabbit plasma HPRG migrates in SDS/PAGE with an apparent molecular mass of 90 or 94 kDa (4, 18, 19) higher than that deduced from its sequence (58 kDa) (4). Inter-
Zinc-binding Site of Skeletal Muscle HPRG

3179

Purification of rabbit skeletal muscle AMP deaminase: elution of the enzyme held by cellulose phosphate after the washing step with 0.45 M KCl

The results are the average values of the determinations carried out on two different AMP deaminase preparations. In each, the three protein fractions were obtained by following the two different elution procedures of the cellulose phosphate-adsorbed enzyme prepared from the muscle of the same rabbit.

| One-step elution with 1.0 M KCl | Two-step elution |
|--------------------------------|------------------|
| Total protein (mg/100 g of muscle) | 8.5 ± 1.6 | 1.0 ± 0.1 |
| Activity (units/mg) | 170 | 0.5 |
| A_{260}/A_{280} | 1.6 | 1.2 |

To ascertain whether the catalytic subunit of AMP deaminase was present only in traces in peak 1 in Fig. 1, as indicated by the determination of the enzyme activity, we applied a sample of pool 1, denatured by overnight dialysis against 0.5 M NaCl, 20 mM sodium phosphate buffer, pH 7.0, containing 0.1% β-mercaptoethanol and 3 M urea, to a metal affinity column (Chelating Fast Flow Sepharose; Amersham Biosciences) charged with Zn²⁺ and equilibrated with the dialysis buffer (results not shown). We have previously used zinc affinity chromatography to separate the 95-kDa HPRG component from the 85- and 70-kDa components present in whole AMP deaminase because the 85- and 70-kDa species are not retained by this resin and eluted in the void volume (20). In contrast, all of the protein present in the urea-denatured sample of pool 1 was retained by the resin and was eluted only when the resin was washed with the EDTA-containing buffer, which strips the metal ions from the gel. Analysis by SDS/PAGE of the eluted fractions revealed identity of migration with the 95-kDa peptide used as starting material. Electroblotting and sequencing of the EDTA-eluted peptide revealed the sequence LPTPTDXKT-LKPLAELDLKI, corresponding to the rabbit plasma HPRG N-terminal sequence (4).

The presence of HPRG as an apparently single component in peak 1 (Fig. 1) indicates that 0.6 M KCl selectively elutes the HPRG component from the AMP deaminase complex adsorbed to cellulose phosphate. However, a significant amount of HPRG is still present as the 85-kDa component in the 1 M KCl-eluted enzyme because the same N-terminal sequence (LPTPTDXKT) shown by the 95-kDa band was obtained by electroblotting and sequencing of the 70-kDa band; furthermore its yield increased with time of storage of the enzyme at 4 °C. A plausible interpretation of this data is that the 70-kDa band contains a C-terminally truncated version of HPRG. This is in agreement with the analysis of the peptides liberated by limited proteolysis of plasma HPRG and of the HPRG component of AMP deaminase showing that both proteins behave as approximately 70-kDa fragments when they are split inside the disulfide bridge connecting the N-terminal domain to the C-terminal domain of the molecule (3, 4). On this basis, we may reasonably assume that on SDS/PAGE of the 1 M KCl-eluted enzyme under reducing conditions, the HPRG component of AMP deaminase and the catalytic subunit both migrate as 85-kDa species, this observation being probably because of an interaction between the two proteins that further reduces the approachability of β-mercaptoethanol to that disulfide bridge.

To establish the extent of the diminution of HPRG content occurring in AMP deaminase as a consequence of the introduction of the washing step with 0.6 M KCl in the phosphocellulose chromatography before the elution of the enzyme with 1 M KCl, we compared the yield of the protein obtained from the skeletal muscle of the same rabbit by following either that protocol or the direct elution with 1 M KCl after the washing step with 0.45 M KCl. The total protein yield in terms of mass was about the same with the two methods, because the protein eluted successively with 0.6 and 1.0 M KCl accounted for about 15 and 85%, respectively, of the total protein eluted with 1.0 M KCl as a single step (Table I). Taking into account the 85-kDa molecular mass of the catalytic subunit of AMP deaminase and assuming for the AMP deaminase associated HPRG the same molecular weight of 58-kDa calculated for the rabbit plasma protein (4), these data are consistent with an approximate 30% diminution of the HPRG content of the phosphocellulose-bound enzyme as consequence of the washing step with 0.6 M KCl. The enzyme obtained with the one-step elution with 1.0 M KCl showed a 20% lower specific activity and a lower A_{260}/A_{280} ratio, indicating a possible contamination by nucleic acids or nucleotides. By following the procedure described under “Experimental Procedures” for the determination of the A_{260}^1% cm value, the high value of 12.6 was calculated for this enzyme, in comparison with that of 9.1 obtained for the enzyme purified adopting the 0.6 M KCl washing step. Altogether, these data indicate that elution with 0.6 M KCl of the AMP deaminase adsorbed to cellulose phosphate probably removes from the resin a protein-protein complex with an extremely high HPRG/AMP deaminase molar ratio, thereby increasing the specific activity of the enzyme isolated in the successive elution of the column with 1.0 M KCl. It should be noted that AMP deaminase prepared as described in Ref. 15 from frozen rabbit skeletal muscle showed an A_{260}^1% cm value of 9.1 (21). Analysis by sedimentation-equilibrium techniques revealed that that enzyme had a molecular weight of 278,000 (21), somewhat lower than that calculated for a molecular aggregate of four identical 85-kDa subunits. This observation was previously explained with the finding that freezing of the muscle causes the same 85–70 kDa transition observed with aging of the purified enzyme (8). In contrast, our determination by sedimentation-equilibrium analysis of the molecular mass of freshly prepared rabbit skeletal muscle AMP deaminase in 1 M KCl, pH 7.0, indicated the presence of two species of 173 and 309 kDa, which were interpreted as being consistent with the existence of a dimer-tetramer equilibrium (22). In the light of the data of the present paper, the heterogeneity observed in sedimentation-equilibrium centrifugation
of the native enzyme should be interpreted as being due to the presence of HPRG/AMP deaminase protein-protein complexes with different molar ratio, the observed 309-kDa molecular mass determined for the heavier component being in agreement with a model for AMP deaminase quaternary structure in which two 85-kDa catalytic subunits assemble with two approximately 70-kDa HPRG subunits (assuming a carbohydrate content similar to that of the plasma protein).

As far as the effect of the diminution of the HPRG content in the preparation of AMP deaminase on the properties of the enzyme is concerned, comparison of the results obtained with the enzymes prepared by following the two different protocols has not given any evidence of clear differences in the kinetics (results not shown) or in the behavior on SDS/PAGE. However, the HPRG-enriched enzyme showed an apparent reduction in the rate of the proteolytic phenomena with storage; it is evident from Fig. 2C (lanes 1–5) that both the disappearance of the 95-kDa band and the 85–70-kDa band transition are significantly slower for the HPRG-enriched enzyme. Our sequence data show that the HPRG variant present in the AMP deaminase preparation shares with the plasma protein an almost totally conserved cystatin domain at the N terminus (23), diverging only at one amino acid residue among the 46 sequenced up to now\(^2\); therefore, we suggest a protective role for the HPRG component against the protease-induced fragmentation of AMP deaminase in addition to its recently suggested action in assuring the molecular integrity of the enzyme (20).

It has been established (5, 6) that HPRG from rabbit serum binds Hg\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Cd\(^{2+}\), and Co\(^{2+}\) in descending order of binding affinity. To obtain a preliminary indication of specific versus nonspecific binding of zinc to the muscle specific form of HPRG, the interaction of the protein with metals was assessed directly by monitoring changes in UV absorption. When compared with HPRG alone, the protein in the presence of increasing equivalents of Zn\(^{2+}\), Cu\(^{2+}\), or Ni\(^{2+}\) showed an increase in absorbance around 275 nm (Fig. 3), a feature consistent with the interaction altering the environment of tyrosine and phenylalanine residues. The change in absorbance at 275 nm produced by each of the three metals showed a maximum when the HPRG samples contained near 40 equivalents of metal ions. Further additions of each metal up to 50 metal ions/HPRG molecule caused only minor changes in the spectra of HPRG that were almost superimposable to those obtained at 40 metal ions/protein. At metal/HPRG ratios higher than 50, different perturbation of the ultraviolet absorption spectrum of HPRG was observed with a decrease rather than an increase of the absorbance at 275 nm. These data may be interpreted as being due to the existence of competing effects (high affinity binding causing an increase in absorbance and low affinity binding causing a decrease in absorbance). However, because an almost constant value of the maximum \(A_{275}\) was determined in the range 40–50 metal ions/protein before the second perturbation became evident, we adopted that value as representing the apparent saturation of the sites with higher affinity.

Titrations of the interaction of HPRG with Zn\(^{2+}\) or Ni\(^{2+}\) shows a sigmoidal relationship (Hill coefficient, \(h = 4.0\) and 3.5, respectively), indicating that these metals are bound in a cooperative manner with interactions among various sites (Fig. 3). Cu\(^{2+}\) binds to HPRG with the same apparent stoichiometry observed with Zn\(^{2+}\) and Ni\(^{2+}\) but shows a minor positive cooperativity of binding (\(h = 1.5\)).

Our results are in partial agreement with the previously reported effects of metals on the fluorescence of rabbit plasma HPRG (5), showing that HPRG interacts with various divalent metal ions with an apparent stoichiometry of 10 (Cu\(^{2+}\), Co\(^{2+}\), Ni\(^{2+}\), and Hg\(^{2+}\)) to 20 (Zn\(^{2+}\) and Cd\(^{2+}\)) metal ions bound per HPRG molecule. The existence of some nonoverlapping sites was suggested by the sigmoidal binding of Zn\(^{2+}\) and Cd\(^{2+}\) (\(h = 4.4\) and 2.8, respectively), whereas the other metals were bound in a noncooperative manner. Moreover, only Zn\(^{2+}\) and Cd\(^{2+}\) of increasing equivalents of HPRG alone, (dashed line), or in the presence of increasing (15–80 \(\mu\text{M}\)) concentrations of ZnCl\(_2\) (solid lines), or in the presence of 100 \(\mu\text{M}\) ZnCl\(_2\) (dotted line). The inset shows the fractional saturation (\(a\)) of HPRG with increasing amounts of ZnCl\(_2\). NiCl\(_2\) (○), or CuCl\(_2\) (●). In each case, the concentration of HPRG in 0.2 M KCl, 5 mM Na\(_2\)HPO\(_4\), pH 8.0, was 1.6 \(\mu\text{g}\), and the increase in absorbance at 275 nm was used to determine \(a\).

\(^2\) A. J. G. Moir and A. Raggi, unpublished work.
caused an enhancement rather than a quenching of fluorescence (5). Altogether, these results were interpreted as an indication that all of the metal ions probably share the same set of binding sites, although the interaction of each metal with the protein may vary because of the involvement of a different group of protein ligands in a different way. This interpretation, which can be extended to the data of protein-metal interactions we have obtained with the muscle-specific form of HPRG, permits one to conclude that most of such a large number of metal-binding sites are nonspecific and that most of the zinc is simply adventitiously bound. Therefore, to minimize the effect of a distribution of zinc-binding sites and to enhance the chance of characterizing the structure of a specific binding site, our investigation by XAS of the zinc binding behavior of HPRG was carried out with a sample obtained by adding only 2 equivalents of zinc to the protein. The usefulness of this approach was confirmed by the inspection of XAS data obtained with HPRG samples containing 8 or 15 equivalents of zinc (results not shown). In these spectra, different EXAFS patterns were superimposed on the spectrum reported in the present paper, preventing a thorough analysis.

X-ray Absorption Spectroscopy—The full-length cDNA sequence of plasma human and murine HPRG and a partial cDNA sequence of the rabbit protein have been reported (2, 4, 24). Alignment of the predicted amino acid sequences indicates that all HPRG species share an overall domain structure comprising an N-terminal domain containing two cystatin-like repeats, a histidine-proline-rich domain, and a C-terminal domain. Alignment of our partial amino acid sequence data of the rabbit skeletal muscle AMP deaminase-associated variant of HPRG with the predicted sequence of rabbit plasma HPRG indicates that the rabbit muscle and plasma forms of HPRG share high identity, diverging only at 5 amino acid residues among the 78 sequenced (93.6% identity) that span all the three proposed overall domains of HPRG.2

The 34 histidine residues located in the histidine-proline-rich domain of rabbit plasma HPRG have been shown to mediate interactions with transition metals, although no evidence has been given of the existence of any specific metal-binding site (4). Because the HPRG component of rabbit skeletal muscle AMP deaminase contains 10 mol of histidine residues/10,000 g of protein (3), we exploited metal affinity chromatography in an attempt to separate the novel component of the enzyme from the catalytic subunit. The isolation of the HPRG component from the purified enzyme under denaturing conditions was achieved by zinc affinity chromatography (20). The observation of the present paper that under nondenaturing conditions the HPRG component can be partially dissociated at a high degree of purity from cellulose phosphate-bound AMP deaminase by an one-step elution with 0.6 M KCl prompted us to utilize this procedure to obtain an HPRG sample suitable for investigation by XAS of the zinc-binding behavior of the protein.

The zinc-K-edge region of the sample is reported in Fig. 4, whereas the EXAFS spectrum and the FT are reported in Fig. 5 (panels a and b, respectively). The presence of the characteristic camelback features in the EXAFS spectrum denotes histidine binding to zinc. In addition, the EXAFS shows beat nodes at about 9.0 and 10.5 Å⁻¹ that evidence destructive interference of the signal caused by the existence of different backscatterer distances in the zinc coordination. These features render the simulation of the spectrum quite difficult, and several different models of the zinc coordination in HPRG were attempted before a satisfactory fit was obtained. Table II reports the structural parameters relative to the zinc coordination in the HPRG protein obtained from the best fit to the spectrum.

Zinc-binding Site of Skeletal Muscle HPRG

Fig. 4. Normalized zinc edge region of the zinc-HPRG 2:1 complex.

Fig. 5. a, EXAFS spectrum (solid line) of the zinc-HPRG 2:1 complex; b, Fourier transform (solid line) superimposed to the best fit (open circles) obtained with the parameters reported in Table II. The phase of the FT is calculated from the first shell atom backscattering factors.

The presence of different bond lengths in the zinc coordination polyhedron is evident from the EXAFS Fourier transform (Fig. 5b) where the main feature shows two closely spaced maxima corresponding to zinc ligand distances of about 2.0 and
Zinc-binding Site of Skeletal Muscle HPRG

2.3 Å. Although the 2.0 Å distance is typical of oxygen/nitrogen donors, the 2.3 Å distance suggests the presence of heavier do-nor atoms in the zinc first coordination shell. All possible different combinations of oxygen/nitrogen-His donors summing up to 4, 5, and 6 were tested to model the 2.0 Å shell, whereas sulfur, chlorine, and zinc backscatterers were tried to model the 2.3 Å shell. It was immediately evident that zinc backscatter-ing could not be the cause of the outer distance shell that was, on the contrary, easily modeled by one sulfur ligand. An identical fit was obtained by replacing sulfur with a chlorine atom at a slightly shorter distance (Table II). Introduction of multiple scattering effects from histidine imidazole rings was able to fully reproduce the spectrum. The best EXAFS and FT fit results in an average zinc coordination polyhedron composed of 3 nitrogen atoms from histidine residues at 1.99 Å and 1 sulfur ligand at 2.28 Å (Cl at 2.25 Å). Besides the peaks at about 3.0 and 4.2 Å, caused by multiple scattering from the histidine second and third shell atoms, the spectrum FT shows a peak at about 3.6 Å (Fig. 5b). The position of this peak does not change if the FT is performed over a different k range, and it is present also in the FT of the EXAFS spectrum of a HPRG-zinc complex collected at a lower HPRG concentration (100 μM) and a higher zinc-HPRG ratio (8:1 and 15:1; data not shown), suggesting that this peak is not an artifact but a real component of the HPRG spectrum. The 3.6 Å FT peak could easily be reproduced in the fit by assuming the presence of a second zinc ion at this distance. On the contrary, this feature could not be reproduced by fitting a shell of four or five carbon atoms between 3.5 and 3.7 Å. Although perfectly reproducing the FT of the spectrum, the inclusion of the zinc shell at 3.68 Å only slightly improves the fit R-factor (2%). The unambiguous detection of metal-metal scattering at distances larger than 3.0 Å in protein EXAFS is always difficult (25), because many factors like multiple scattering from other ligands, metal carbon scattering, etc., usually interfere with the metal-metal scattering (25). However, the example of the EXAFS spectrum of urease from jack bean, Klebsiella aerogenes and Bacillus pasteurii (26, 27) are particularly instructive because the spectra FTs of both native and β-mercaptoethanol-inhibited enzymes always show an outer shell pattern identical to the HPRG-Zn2 complex with a peak at about 3.5 Å in between the histidine outer shell peaks. A dinuclear Ni2+ cluster is present in urease with nickel-nickel distances ranging between 3.1 and 3.5 Å (26–28). In summary, although not conclusive, the evidence from our EXAFS data suggests that the zinc-binding site in HPRG may host two metal ions at a distance of about 3.7 Å.

On average the zinc ion in HPRG appears to be 4-coordinated. However, it is known that coordination numbers ob-
2.23–2.25 Å, but a thiolate sulfur seems the most probable candidate.

The EXAFS analysis of the 2:1 zinc-HPRG complex shows that the protein is able to bind zinc, possibly in a dinuclear metal-binding site where two Zn$^{2+}$ ions are bound to histidine residues. In principle, the EXAFS analysis gives only the average zinc coordination in the sample and does not establish whether one zinc ion is in a histidine only environment (i.e. zinc-N(His)$_5$) and the other in a nitrogen/sulfur coordination (i.e. zinc-N(His)$_3$S$_2$) or whether the zinc-binding site is a more symmetric one with common bridging ligands. However, the zinc-zinc distance of 3.68 Å in HPRG is typical of dinuclear first transition row metal sites with bridging ligands like carboxylates or cysteine thiolate sulfur. Examples of dinuclear zinc sites can be seen in dithioretic esterase where two Zn$^{2+}$ ions, bridged by the carboxylate group of a carbamylated Lys residue, are at 3.48 Å distance. This also occurs in phosphotriesterase where two Zn$^{2+}$ ions are at 3.45 Å (35). In β-mercaptoethanol inhibited urease (Protein Data Bank entry 1UBP), the two Ni$^{2+}$ ions in the active site are bridged by the inhibitor thiolate sulfur bringing them as close as 3.1 Å (28). Possible models of the zinc site found in HPRG that are consistent with the structural data obtained from EXAFS are reported in Fig. 6.

The finding that the XAS data support the presence of a dinuclear cysteine-bridged zinc-binding site in the HPRG molecule dissociated from rabbit skeletal muscle AMP deaminase deserves consideration in light of the determination of the disulfide bridge arrangement of bovine plasma HPRG that has shown that all the 12 half-cysteine residues found in the protein are involved in the formation of six disulfide bridges (36).

Alignment of the partial amino acid sequence data of bovine plasma HPRG with the available predicted amino acid sequences of human, mouse, rat, and rabbit HPRG indicates that 11 of the 12 cysteine residues are totally conserved in all species, suggesting that five disulfide bridges are likely to be essential for the proper folding of the protein of each species. The two cysteine residues that in bovine HPRG form the disulfide bond within one of the two proline-rich regions that flank the histidine-rich region are conserved in rabbit HPRG (residues 264 and 294) but not in the human protein (Cys-264 of the rabbit HPRG sequence is replaced by proline) and in mouse and rat HPRG, where Cys-294 is absent. It should be noted that a number of other cysteine residues are present at different positions in the histidine-proline-rich domain of human, mouse, and rat HPRG. This analysis suggests that the cysteine residue that is not involved in disulfide bond formation and that is therefore the candidate for zinc ligation in the muscle variant of HPRG as suggested by EXAFS belongs to this region of the protein. The same conclusion is likely to be true for the six histidine residues used by the protein as zinc ligands, because it was shown that one isolated 30-kDa His-Pro-Gly-rich peptide retains the ability of rabbit plasma HPRG to bind metals (18).

The characterization of skeletal muscle AMP deaminase as a zinc metalloenzyme was reported for the rat enzyme (37) and for the rabbit enzyme (21) on the basis of its interaction with chelating agents and metal ions. Overall, the results of the present study clearly indicate the presence in HPRG of a zinc-binding site and permit the assertion that the two components of AMP deaminase manifest as a common property the ability to interact with zinc ions. The stoichiometry of zinc binding to rabbit skeletal muscle AMP deaminase as found in vivo has yet to be absolutely established (21), because the native enzyme as isolated, which our results indicate to be made up of the catalytic subunit and the HPRG component, contains 2.6 g atoms of zinc/mol (molecular mass, 278 kDa), whereas the apoenzyme binds 4 g atoms of zinc/mol. However, the increase of $V_{\text{max}}$ caused by the addition of the fourth zinc atom is only 28% of that expected. This suggests that the fourth zinc atom is not directly associated with activity (21).

Alignment of the amino acid sequence for yeast AMP deaminase with that for mouse adenosine deaminase demonstrates conservation of the four amino acids (three His and one Asp) known from the x-ray crystal structure of adenosine deaminase to bind zinc in contact with the attacking water nucleophile (38). On the basis of these similarities, the same model of a pentacoordinated zinc bound at the catalytic site that was described for adenosine deaminase, a 352-amino acid protein, has also been proposed for the 810-amino acid monomer of yeast AMP deaminase (39). However, we may point out that alignment of the amino acid residues supposed to be in contact with zinc in yeast AMP deaminase with the deduced amino acid sequence for the skeletal muscle enzyme demonstrates conservation of only three amino acids (His-363, His-572, and Asp-649, corresponding respectively to His-422, His-630, and Asp-707 in yeast AMP deaminase), whereas residue 424 of the yeast enzyme sequence (His) is replaced by Gly-365 in both rat and human skeletal muscle enzymes. Altogether, these observations confirm that zinc is a firmly bound component of skeletal muscle AMP deaminase essential for the enzyme activity but also suggest that the model of zinc-binding site proposed for yeast AMP deaminase cannot be unambiguously extended to the skeletal muscle enzyme.

The separation of HPRG using zinc affinity chromatography under denaturing conditions induced a marked reduction in the solubility of the catalytic subunit of skeletal muscle AMP deaminase, strongly suggesting an additional role for HPRG in the maintenance of the native quaternary structure of the enzyme that could be envisaged from the formation of a 1:1 HPRG-AMP deaminase molecular adduct (20). The observation of the present paper that with the HPRG-enriched enzyme the rate of the fragmentation of AMP deaminase with storage is reduced (Fig. 2) suggests another possible role of the HPRG component in preserving the molecular integrity of the enzyme. It seems to be premature to assign a physiological role to the additional zinc not required for activity (21) with the finding of the present paper that with the HPRG-enriched enzyme the rate of the fragmentation of AMP deaminase with storage is reduced (Fig. 2) suggests another possible role of the HPRG component in preserving the molecular integrity of the enzyme. It seems to be premature to assign a physiological role to the additional zinc not required for activity (21) with the finding of the present paper that with the HPRG-enriched enzyme the rate of the fragmentation of AMP deaminase with storage is reduced (Fig. 2) suggests another possible role of the HPRG component in preserving the molecular integrity of the enzyme. It seems to be premature to assign a physiological role to the additional zinc not required for activity (21) with the finding of the present paper that with the HPRG-enriched enzyme the rate of the fragmentation of AMP deaminase with storage is reduced (Fig. 2) suggests another possible role of the HPRG component in preserving the molecular integrity of the enzyme. It seems to be premature to assign a physiological role to the additional zinc not required for activity (21) with the finding of the present paper that with the HPRG-enriched enzyme the rate of the fragmentation of AMP deaminase with storage is reduced (Fig. 2) suggests another possible role of the HPRG component in preserving the molecular integrity of the enzyme. It seems to be premature to assign a physiological role to the additional zinc not required for activity (21) with the finding of the present paper that with the HPRG-enriched enzyme the rate of the fragmentation of AMP deaminase with storage is reduced (Fig. 2) suggests another possible role of the HPRG component in preserving the molecular integrity of the enzyme. It seems to be premature to assign a physiological role to the additional zinc not required for activity (21) with the finding of the present paper that with the HPRG-enriched enzyme the rate of the fragmentation of AMP deaminase with storage is reduced (Fig. 2) suggests another possible role of the HPRG component in preserving the molecular integrity of the enzyme. It seems to be premature to assign a physiological role to the additional zinc not required for activity (21) with the finding of the present paper that with the HPRG-enriched enzyme the rate of the fragmentation of AMP deaminase with storage is reduced (Fig. 2) suggests another possible role of the HPRG component in preserving the molecular integrity of the enzyme. It seems to be premature to assign a physiological role to the additional zinc not required for activity (21) with the finding of the present paper that with the HPRG-enriched enzyme the rate of the fragmentation of AMP deaminase with storage is reduced (Fig. 2) suggests another possible role of the HPRG component in preserving the molecular integrity of the enzyme. It seems to be premature to assign a physiological role to the additional zinc not required for activity (21) with the finding of the present paper that with the HPRG-enriched enzyme the rate of the fragmentation of AMP deaminase with storage is reduced (Fig. 2) suggests another possible role of the HPRG component in preserving the molecular integrity of the enzyme.
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