Association of purified skeletal-muscle AMP deaminase with a histidine–proline-rich-glycoprotein-like molecule

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Denaturation of rabbit skeletal-muscle AMP deaminase in acidic medium followed by chromatography on DEAE-cellulose in 8 M urea at pH 8.0 allows separation of two main peptide components of similar apparent molecular mass (75–80 kDa) that we tentatively assume correspond to two different enzyme subunits. Whereas the amino acid composition of one of the two peptides is in good agreement with that derived from the nucleotide sequence of the known rat and human AMPD1 cDNAs, the second component shows much higher contents of proline, glycine and histidine. N-Terminal sequence analysis of the fragments liberated by limited proteolysis with trypsin of the novel peptide reveals a striking similarity to the fragments produced by plasmin cleavage of the rabbit plasma protein called histidine–proline-rich glycoprotein (HPRG). However, some divergence is observed between the sequence of one of the fragments liberated from AMP deaminase by a more extensive

trypsinization and rabbit plasma HPRG in the region containing residues 472–477. A fragment with a blocked N-terminus, which was found among those liberated by proteolysis with pepsin of either whole AMP deaminase or the novel component of the enzyme, shows an amino acid composition quite different from that of the N-terminus of the known subunit of AMP deaminase. By coupling this observation with the detection in freshly prepared AMP deaminase of a low yield of the sequence (LTPTDX) corresponding to that of HPRG N-terminus, it can be deduced that in comparison with HPRG, the putative HPRG-like component of AMP deaminase contains an additional fragment with a blocked N-terminus, which is liberated by a proteolytic process during purification of the enzyme. The implications of the association to rabbit skeletal-muscle AMP deaminase of a HPRG-like protein species are discussed.

INTRODUCTION

In a previous paper [1] we showed by using SDS/PAGE that AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) isolated from fresh rabbit skeletal muscle gave rise to a major 79 kDa protein band, termed α , and a minor 73 kDa protein band, termed α' . After limited proteolysis with trypsin, the original 79 kDa band disappeared and only one band of approx. 72 kDa was found. The apparent α -to- α' transition also occurred on storage of AMP deaminase, as well as of the muscle used for the enzyme preparation. In every case, when the conversion was complete the electrophoretic properties of the enzyme did not vary on further storage, indicating that the α' subunit was resistant to proteolysis.

In a recent paper we gave evidence that limited cleavage by trypsin removes from AMP deaminase a 10 kDa N-terminal fragment that encompasses a non-catalytic regulatory domain [2]. Analysis by sedimentation–equilibrium techniques revealed the proteolysed enzyme to be homogeneous and to have a trimeric structure, in contrast with the tetrameric structure of the native enzyme [3]. Although these observations seemed to confirm the data of the literature documenting that skeletal-muscle AMP deaminase is composed of four identical subunits [4–11], comparison of amino acid composition of the native enzyme with that derived from the nucleotide sequences of rat and human skeletal-muscle AMP deaminase (AMPD1) cDNAs [12] revealed important and significant differences.

We report here the isolation, from purified rabbit skeletal-muscle AMP deaminase, of an approx. 75 kDa novel peptide

with an amino acid composition significantly different from that derived from the available cDNAs nucleotide sequences. N-Terminal sequence analysis of the fragments liberated by limited proteolysis revealed a striking similarity of this protein to the rabbit plasma protein called histidine–proline-rich glycoprotein (HPRG) [13].

MATERIALS AND METHODS

Materials

Trypsin, pepsin, carboxypeptidase A and B and bovine ribonuclease were purchased from Sigma. Sephadex G-15 and G-50 were from Pharmacia. SP-Trisacryl was from LKB. ZeoKarb 225 was from Koch-Light Laboratories. Problott PVDF and all sequence reagents were purchased from Applied Biosystems. The other reagents used were of analytical grade.

Native and trypsin-cleaved AMP deaminases

AMP deaminase was prepared as described previously from fresh muscle dissected from the back and hind leg of rabbits [14].

Limited proteolysis of AMP deaminase with trypsin at pH 7.0, and separation of the proteolysed enzyme from trypsin and by-products were carried as described in ref. [15]. The enzyme concentration was calculated from the absorbance at 280 nm assuming an $A^{1\%,1\text{cm}}$ value of 9.13 [16]. Spectrophotometric determinations were carried out in a Shimadzu UV-260 spectrophotometer.

Abbreviations used: HPRG, histidine–proline-rich glycoprotein.

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Separation of the components of AMP deaminase

AMP deaminase solution (3–5 ml; 2.7 mg/ml) was dialysed overnight against 6 M urea/5 mM 2-mercaptoethanol/0.9 M acetic acid (pH 3.2) and then against two changes of 8 M urea/5 mM 2-mercaptoethanol/50 mM Tris/HCl buffer (pH 8.0) at room temperature. The dialysed enzyme was applied under gravity to a column (2 cm × 36 cm) of DEAE-cellulose (Whatman DE52) equilibrated with 8 M urea/5 mM 2-mercaptoethanol/50 mM Tris/HCl buffer (pH 8.0). Starting buffer (250 ml) was then pumped on to the column at a rate of 30 ml/h, followed by application of a gradient of 0–0.6 M NaCl in the above buffer. Protein peaks were pooled and dialysed against 5 mM HCl. After five changes of dialysis fluid the fractions were freeze-dried and stored at –20 °C.

Fractionation of limited digests of AMP deaminase

Limited proteolysis of AMP deaminase (13.5 mg) with trypsin (0.09 mg) was carried out in the presence of 1 M KCl adjusted to pH 6.0 with 0.1 M KH_2PO_4 (20 °C). After 5 min the reaction mixture was passed through a column of Sephadex G-50 (2.4 cm × 140 cm) equilibrated and eluted with 10 mM HCl. Fractions of 5 ml were collected and the elution of peptides was followed by measuring A_{215} and A_{280} .

More extensive trypsinization of AMP deaminase (0.8 mg) with trypsin (0.01 mg) was carried out in the presence of 1 M KCl adjusted to pH 7.0 with 1 M K_2HPO_4 (20 °C). After 60 min the digestion was stopped by addition to the reaction mixture of 1 μl of 1 M HCl, which caused protein precipitation. The constituent peptides of the supernatant were separated by reversed-phase HPLC using an Alphasil C18 column equilibrated with 0.1% (v/v) trifluoroacetic acid. Peptides were eluted using a gradient, at 1 ml/min, of acetonitrile containing 0.1% trifluoroacetic acid/0–21% (v/v) acetonitrile over 2 min, followed by 21–28% acetonitrile over 18 min and 28–70% acetonitrile over 17 min. The HPLC apparatus consisted of a Model 600 E from Waters Associates.

Limited proteolysis of AMP deaminase (5 mg) with pepsin (0.05 mg) was carried out in the presence of 1 M KCl adjusted to pH 6.0 with 0.1 M KH_2PO_4 (20 °C). After 1 h the reaction mixture was passed through a column (1 cm × 45 cm) of Sephadex G-15 equilibrated and eluted with water. Fractions of 0.8 ml were collected and their A_{280} was measured. The fractions that were pooled were freeze-dried and stored at –20 °C.

PAGE

Electrophoresis in the presence of 0.1% (w/v) SDS was carried out on 8% (w/v) polyacrylamide slab gels in 0.1 M Tris/0.1 M Bicine (pH 8.3). Electrophoresis was also carried out in 6 M urea/0.9 M acetic acid (pH 3.2), as described by Panyim and Chalkley [17].

C-Terminal sequence investigations

The C-terminal amino acids of native AMP deaminase, its separated components and trypsinized enzyme were investigated by digestion with carboxypeptidase A, carboxypeptidase B and a mixture of the two, over a wide range of enzyme/substrate ratios and digestion times. Samples containing up to 0.2 mg of AMP deaminase were digested in the presence of 0.7 M KCl and 35 mM NaHCO_3 with as much as 0.2 nmol of carboxypeptidase at 20 °C for as long as 4 h. Denaturation of substrate was accomplished by treatment with 2% SDS at 100 °C. Released

amino acids were identified and quantified by HPLC after labelling with dansyl chloride. Bovine ribonuclease was treated under identical conditions.

Electroblotting and amino acid and sequence analysis

Electroblotting was performed by the method of LeGendre and Matsudaira [18] using 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid containing 10% (v/v) methanol. Transfers were performed for 60–90 min at 400 mA.

Amino acid analysis was performed with a Multichrom B Beckman automatic analyser according to Moore and Stein [19], on protein samples (0.4–1.0 mg) hydrolysed at 110 °C in evacuated, sealed tubes in HCl (6 M, 0.4–1.0 ml) for 24 and 72 h. Serine and threonine data were extrapolated to zero time. Amino acid analysis was also performed using a Waters Picotag system operated under standard conditions. N-Terminal sequencing was performed using an Applied Biosystems model 476A protein sequencer.

RESULTS

Amino acid composition of rabbit skeletal-muscle AMP deaminase

The amino acid compositions of native and trypsinized rabbit skeletal-muscle AMP deaminases are shown in Table 1 compared with those derived from the nucleotide sequence of human and rat cDNAs.

Table 1 Amino acid composition of rabbit skeletal-muscle AMP deaminase

Figures are given as mol of amino acid/10000 g of protein. Amino acid contents were determined for intact AMP deaminase and for the trypsin-cleaved enzyme separated from trypsin and by-products as previously described [15]. Results are based on duplicate analyses of two preparations of native as well as trypsinized enzyme. For comparison, the first column reports the average amino acid values calculated from the data of the literature [6,11,20]. The last two columns represent the amino acid compositions deduced from the sequence of rat and human AMPD1 cDNAs [12].

Residue	Literature	Amino acid composition (mol of amino acid/10000 g of protein)			
		The present study		cDNA deduced AMPD1	
		Trypsin-treated enzyme	Native enzyme	Human	Rat
Asp	9.1	9.1	8.0	9.8	9.7
Thr	4.4	4.2	4.1	4.2	3.5
Ser	6.2	5.7	5.9	5.8	5.9
Glu	9.1	8.9	9.0	9.0	8.7
Pro	4.9	6.5	6.6	4.9	5.1
Gly	4.0	4.2	4.5	3.3	4.0
Ala	5.4	5.2	6.6	5.1	5.0
Cys	1.2	1.3*	1.6*	1.4	1.4
Val	4.1	4.5	4.4	4.5	4.2
Met	2.6	2.4	1.8	3.0	3.3
Ile	3.8	3.6	3.5	4.3	4.5
Leu	8.2	8.4	7.9	7.9	7.7
Tyr	4.2	4.4	3.6	4.3	4.4
Phe	4.7	4.6	4.4	5.2	5.3
His	2.9	3.5	3.7	2.9	2.9
Lys	7.2	6.0	7.8	6.5	5.9
Arg	4.3	4.2	4.1	3.9	4.4
Trp	0.7	0.8†	0.8†	0.7	0.6

* Determined as cysteic acid in separate samples oxidized with performic acid according to Moore and Stein [19], and then hydrolysed for 24 h.

† Determined spectrophotometrically [21].

Figure 1 Fractionation of AMP deaminase components by chromatography on DEAE-cellulose

A solution (3 ml) ($A_{280} = 2.6$) of AMP deaminase isolated from rabbit skeletal muscle and dialysed against 6 M urea/5 mM 2-mercaptoethanol/0.9 M acetic acid (pH 3.2) and then against 8 M urea/5 mM 2-mercaptoethanol/50 mM Tris/HCl buffer (pH 8.0) was applied to a column (2 cm $\times$ 36 cm) of DEAE-cellulose (DE52) equilibrated with the last buffer. A linear gradient of 0–0.6 M NaCl in the starting buffer was applied at a rate of 30 ml/h. Fractions of 2 ml were collected and A_{280} (—) and A_{260} (---) were measured. Horizontal bars indicate the fractions that were pooled. (---) NaCl concn.

The composition of trypsinized AMP deaminase (with the exception of a higher content of proline and histidine and a lower content of lysine) is similar to that reported in the literature [6,11,20] for the enzyme (subunit molecular mass 69 kDa) prepared from frozen rabbit muscle, which is in keeping with our observation that, on storage, cleavage of the enzyme subunit occurs to give a product similar in size to that obtained on limited proteolysis of AMP deaminase with trypsin [1]. When the composition of native AMP deaminase is compared with that derived from the cDNA nucleotide sequences (subunit molecular mass 85 kDa), [12] a larger number of differences are observed, the enzyme showing a higher content of proline, alanine, histidine and lysine and a lower content of aspartic acid and methionine.

Separation of components

The above observations prompted us to find a procedure to obtain separation of a possible second subunit of AMP deaminase. This was accomplished by enzyme denaturation in acidic medium followed by chromatography on DEAE-cellulose. The enzyme (8 mg) was dialysed overnight against 6 M urea/5 mM 2-mercaptoethanol/0.9 M acetic acid (pH 3.2) and then against two changes of 50 mM Tris/HCl (pH 8.0)/8 M urea/5 mM 2-mercaptoethanol. The dialysed enzyme was loaded on to a column (2 cm $\times$ 36 cm) of DEAE-cellulose equilibrated with the last dialysis buffer. The column was washed with an additional 250 ml of buffer before elution with a linear gradient of 0–0.6 M NaCl in the starting buffer (Figure 1). The protein in the first peak was not retained on the column and emerged in the void volume. Peak 2 appeared at the end of the washing phase, whereas the centres of peaks 3 and 4 were located at 0.1 M and 0.2 M NaCl respectively. Recovery of total material applied to the column in terms of mass of the freeze-dried pools was 5.6 mg (70%), with 16%, 24%, 19% and 41% of this being accounted for by peaks 1, 2, 3 and 4 respectively. Analysis by SDS/PAGE of each pooled peak showed that this method did not distinguish

Figure 2 PAGE at pH 3.2 of AMP deaminase components

Gel conditions were 10% (w/v) polyacrylamide, 6 M urea and 0.9 M acetic acid. (a) 100 μ g of whole AMP deaminase; (b) 50 μ g of pool 1, Figure 1; (c) 50 μ g of pool 2, Figure 1; (d) 50 μ g of pool 3, Figure 1; (e) 50 μ g of pool 4, Figure 1.

Table 2 Amino acid composition of isolated components of AMP deaminase

Figures are given as mol of amino acid/10000 g of protein. Results are the average values of duplicate analyses carried out on two preparations of pools 1–4 obtained with the fractionation described in Figure 1. For comparison, the last column represents the amino acid composition of rabbit plasma HPRG deduced from the sequence of cloned cDNA of this protein [13]. ND, not determined.

Residue	Amino acid composition (mol of amino acid/10000 g of protein)				
	Pool 1	Pool 2	Pool 3	Pool 4	HPRG
Asp	9.1	9.1	10.1	8.8	7.2
Thr	4.1	2.5	3.7	3.6	3.8
Ser	7.4	6.3	7.6	6.0	5.2
Glu	8.2	6.4	7.9	10.6	7.4
Pro	5.4	11.5	6.7	4.5	14.5
Gly	4.9	7.3	5.9	5.5	7.4
Ala	6.1	3.9	4.8	6.4	3.4
Cys	ND	ND	ND	ND	2.1
Val	3.8	2.9	2.9	4.5	4.1
Met	1.9	1.0	0.9	2.5	0.2
Ile	3.5	1.5	2.7	4.4	2.1
Leu	7.5	5.4	5.3	7.4	5.9
Tyr	4.0	3.8	3.2	3.2	1.7
Phe	4.7	5.1	4.8	4.5	5.0
His	4.0	10.0	6.8	2.8	9.1
Lys	7.2	6.7	8.4	7.6	4.1
Arg	4.0	4.0	3.9	4.8	5.5
Trp	ND	ND	ND	ND	0.5

between the components of the four pools, each of them giving a main band of approx. 75–80 kDa (results not shown). A better resolution was obtained with PAGE at pH 3.2 in 6 M urea (Figure 2). Under these conditions, the enzyme used as starting material gave rise to two main bands (Figure 2, lane a), the slower of which was mainly present in the electrophoretogram of pool 1, together with a faster-migrating band (Figure 2, lane b).

Figure 3 Separation by gel filtration on Sephadex G-15 of the peptides liberated by limited proteolysis with pepsin

Limited proteolysis of AMP deaminase (5 mg) with pepsin (0.05 mg) was carried out for 1 h at pH 6.0. The digest was applied to a column (1 cm $\times$ 45 cm) of Sephadex G-15 equilibrated and eluted with water. Fractions of 0.8 ml were collected and A_{280} was measured. Fractions pooled are indicated by the bars.

We may suggest the latter material to be a proteolytic fragment corresponding to the approx. 100-residue-long N-terminus of the enzyme, sometimes detected in the gel of the freshly prepared enzyme and disappearing on storage, or as the effect of limited proteolysis with trypsin (results not shown). Pool 2 contained only one component (Figure 2, lane c), which could be identified as a minor component of the starting material migrating as a double band (Figure 2, lane a). This component was also present in pool 3 (Figure 2, lane d) with a small amount of the main component of pool 4 as a contaminant, corresponding to the faster band shown by the enzyme used as starting material. In addition to this band, pool 4 showed the presence of a small amount of a somewhat faster component (Figure 2, lane e). We may ascribe the latter material to the core fragment produced by the proteolytic phenomenon that removes from AMP deaminase the 95-residue-long N-terminal fragment [2], since on storage or by limited proteolysis with trypsin, this component completely substituted the species corresponding to the main component of pool 4 (results not shown).

The amino acid composition of pools 1, 2, 3 and 4, which are presented in Table 2, accounted for respectively 33 %, 24 %, 6 % and 37 % of the total protein recovered. The low value of absorbance at 280 nm compared with 260 nm shown by fraction 3 (Figure 1) indicates the presence of bound nucleic acid or nucleotide in this fraction. The amino acid composition of pool 1 is similar to that of the native enzyme reported in Table 1. The composition of pool 4 is in agreement with that derived from the nucleotide sequence of rat and human AMPD1 cDNAs (see Table 1). In contrast, the composition of pool 2 differs significantly in many amino acid residues from the predicted composition of AMPD1, especially for much higher contents of proline and histidine.

N- and C-terminus analysis

The above results suggest the presence in the AMP deaminase molecule of two different types of subunits of similar molecular

Table 3 Amino acid composition of blocked N-terminal peptides isolated from AMP deaminase (residues/molecule)

Results are based on duplicate analyses carried out on peptide fractions isolated from digestion mixtures obtained by limited proteolysis of AMP deaminase. Samples I and II refer to peptides not retarded when the fragments liberated by pepsin digestion of native AMP deaminase (pools I and II, respectively, Figure 3) were passed through a ZeoKarb 225 column. Sample III corresponds to the material that did not bind when a peptic digest of the HPRG-like component of AMP deaminase (pool A, Figure 4) was passed through an SP-Trisacryl column. ND, not determined; (–) not detected or trace only.

Residue	Amino acid composition (residues/molecule)		
	Whole enzyme		HPRG-like component
	I	II	III
Asp	2.1	2.5	1.5
Thr	1.3	1.2	0.8
Ser	1.7	2.3	0.9
Glu	2.9	3.3	2.9
Pro	0.9	1.7	0.8
Gly	2.4	2.0	1.5
Ala	2.4	2.0	1.4
Cys	ND	ND	ND
Val	1.5	1.5	1.0
Met	–	0.6	–
Ile	1.0	1.1	1.1
Leu	1.7	2.6	1.4
Phe	0.7	1.4	0.9
Lys	1.4	1.4	1.4
Arg	0.8	1.0	–
Trp	ND	ND	ND

mass. It has been previously reported that attempts to identify an N-terminal residue of rabbit skeletal-muscle AMP deaminase were unsuccessful [2,11], indicating that the N-terminus of the enzyme was modified. Therefore, in order to ascertain whether the putative second subunit of the enzyme is characterized by a primary structure at the N-terminus that is different from that of the sequenced subunit, we followed the method described by Wilkinson [22] for isolation of peptides with a blocked N-terminus.

The peak of fragments liberated and separated from AMP deaminase by the limited proteolysis process with pepsin described in the Materials and methods section was divided into two pools (Figure 3); each of them was passed through a column (0.6 cm $\times$ 8.0 cm) of ZeoKarb 225 (H^+ form). The peptides not retarded by such a column, that were isolated using pools I and II, had the composition (Asx-2, Thr, Ser-2, Glx-3, Pro, Gly-2, Ala-2, Val-2, Ile, Leu-2, Phe, Lys, Arg) and (Asx-2, Thr, Ser-2, Glx-3, Pro-2, Gly-2, Ala-2, Val-2, Met, Ile, Leu-3, Phe, Lys, Arg) respectively (Table 3). The two compositions differ by the presence in pool II of one extra residue of proline, methionine and leucine, which, on the basis of similarity to the N-terminus of human and rat AMP deaminase subunit, suggests that among the fragments removed from AMP deaminase by pepsin, the M-P-L peptide is present in addition to at least another fragment with a blocked N-terminus.

Digestion with carboxypeptidases A and B of either native AMP deaminases or its separated components failed to liberate stoichiometric amounts of any amino acid, in keeping with the observation of Boosman and Chilson [11]. Under the same conditions we were able to successfully identify the C-terminal residue of bovine ribonuclease; this suggests that the C-terminal groups of AMP deaminase components are amidated,

or they are among those residues that are resistant to the exopeptidases. In contrast, arginine was released after 15 min digestion of trypsinized AMP deaminase by carboxypeptidases A and B. Dns-arginine remained the only derivative visible after 30, 60 and 120 min digestion.

N-Terminal sequences of proteolysed AMP deaminase

In a recent paper [2] we reported that all the peptides removed from AMP deaminase by digestion for 5–90 min at pH 7.0 with trypsin (trypsin/AMP deaminase 1:100, w/w) were derived from the N-terminal region up to residue 95. N-Terminal sequence analysis of the 70 kDa peptide isolated by gel filtration after digestion with trypsin gave the sequence L-T-P-I-D-E-Y-I-T, which is similar, but not identical, with residues 96–104 of the rat and human sequences [12]. The above-reported results of this paper, suggesting the enzyme to constitute two different subunits, prompted us to analyse further the core fragment produced by trypsinization of AMP deaminase.

SDS/PAGE of the enzyme after 5–15 min treatment with trypsin, revealed, in addition to the main band of 70–75 kDa, two closely spaced bands of approx. 40 kDa. Whereas electroblotting and sequencing of the 40 kDa double band revealed the sequence L-T-P-T-D-X-K-T-T-K-P-L-A-E, plus a minor sequence H-R-P-F-G, the 70–75 kDa band showed two sequences in equal amount, one of which (L-T-P-T-D-X-K) was identical with that of the two faster-migrating bands, while the other (L-T-H-I-D-E-Y) corresponded to a fragment starting at Leu-96 in the sequenced AMP deaminase subunit. These results strongly suggest that the Pro we reported at position 3 in the N-terminal sequence for the trypsinized enzyme actually resulted from sequencing of the additional component.

Similarity of the novel component of AMP deaminase to plasma HPRG

As described above, three peptides isolated by SDS/PAGE from the tryptic digest of AMP deaminase share the same N-terminal amino acid sequence. A computer search revealed a high degree of similarity between this sequence and the cystatin-like domains belonging to the known members of the cystatin superfamily [23]. In particular, a striking 100% identity with the N-terminal sequence of mature rabbit-plasma HPRG [13] is found in our sequence when a half-cysteine residue is assumed to be present in its sixth position. The suggestion of the presence in AMP deaminase of a HPRG-like component is strengthened by the observation that the amino acid composition of pool 2 obtained with the above-described fractionation of AMP deaminase (Figure 1) is very similar to that obtained by peptide sequencing of rabbit HPRG (Table 2).

When the freshly prepared enzyme was subjected to automated Edman degradation, a low yield of three sequences was obtained, indicating that a small degree of proteolysis had taken place during isolation of the enzyme. Two of them, F-K-L-P-A-E and P-L-F-K-L-P, corresponded to fragments beginning respectively at Phe-4 and Pro-2 of the sequenced subunit and were present as about 5% and 2% of the total enzyme. The third sequence, L-T-P-T-D-X, corresponding to the rabbit HPRG N-terminus, was present in equal amount to that beginning at Pro-2. By coupling this observation with the results of the N-terminus analysis of native AMP deaminase (see above), indicating the presence of a blocked N-terminus with an amino acid composition quite different from that deduced from the available cDNAs sequences, we can deduce that the putative second component of AMP deaminase contains at the N-terminus an additional fragment that is absent in rabbit HPRG.

Figure 4 Fractionation by gel filtration on Sephadex G-50 of the products of a limited AMP deaminase tryptic digest

Limited proteolysis of AMP deaminase (13.5 mg) with trypsin (0.09 mg) was carried out for 5 min at pH 6.0. The digest was applied to a column (2.4 cm $\times$ 140 cm) of Sephadex G-50 equilibrated and eluted with 10 mM HCl. Fractions of 5 ml were collected and A_{215} (—) and A_{280} (---) were measured. Fractions pooled are indicated by the bars.

Isolation of the HPRG-like component of AMP deaminase by gel-filtration chromatography

When freshly prepared AMP deaminase was digested for 5 min at pH 6.0 with a reduced amount of trypsin (trypsin/AMP deaminase 1:150, w/w) the subsequent chromatography of the digest on Sephadex G-50 in 10 mM HCl was affected by a certain amount of protein precipitation. This process altered the filtration of the core fragments produced by the proteolysis so that they were eluted as two separate peaks (Figure 4, pools A and B). The constituent peptides of pool A were separated by SDS/PAGE revealing the presence of single bands of 70 and 30 kDa and two closely spaced bands of approx. 40 kDa. Whereas electroblotting and sequencing of the 30 kDa band revealed a single sequence H-R-P-F-G-T-X-E-T-H-R-F, corresponding to the rabbit HPRG fragment beginning at His-296 [13], each of the other three bands showed two sequences in equal amounts, one of which was identical with the sequence of the 30 kDa fragment and the other, L-T-P-T-D-X-K-T-T-K-P-L-A-E, corresponded to the N-terminal sequence of rabbit HPRG [13]. Sequencing of pool B revealed the presence of approximately equal amounts of several peptides deriving from the cleavage of the N-terminus of the AMP deaminase subunit (at residues 11, 18, 30, 79, 80, 95). N-terminal analysis of the components of the pools containing smaller fragments identified them as fragments 19–73 and 24–73 (pool C) and 2–18, 4–18, 80–95 and 81–95 (pools D and E), all of them deriving from digestion within residues 1–95 of AMP deaminase.

In order to ascertain whether pool A also contained the peptide with a blocked N-terminus as suggested by the above reported results of the N-terminus analysis of the native enzyme, a sample of that pool was digested with pepsin in 10 mM HCl and the digest was passed through SP-Trisacryl in 10 mM HCl (1 cm $\times$ 7 cm). The material that did not bind was freeze-dried, hydrolysed and then run on the Pico Tag system. The amino acid composition of this material matches that obtained

Figure 5 HPLC of the soluble fraction obtained after acid-precipitation of a tryptic digest of AMP deaminase

An Alphasil C18 column was used with the gradient of acetonitrile (---) described in the Materials and methods section. Peaks were isolated and subjected to N-terminal analysis. The figures above and below the identified peptides indicate their collocation in the sequence of rabbit skeletal-muscle AMP deaminase and plasma HPRG respectively.

with the native enzyme with the exception of a lower content of Ser, Ala, Val and Arg (Table 3).

Homology with HPRG of the amino acid sequence of peptides obtained by more extensive trypsinization of AMP deaminase

In order to ascertain whether the observed similarity of the novel component of AMP deaminase to rabbit HPRG could be confirmed by a further characterization of its primary structure, N-terminal sequencing was performed on peptides liberated by a more extensive proteolysis of AMP deaminase.

Freshly prepared AMP deaminase was digested for 60 min at pH 7.0 with trypsin (trypsin/AMP deaminase 1:80, w/w). The addition to the digest (400 μ l) of 1 μ l of 1 M HCl caused protein precipitation, but reversed-phase HPLC of the supernatant revealed the presence of several peptides (Figure 5) that were isolated and subjected to N-terminal analysis. In addition to fragments 4–11, 6–11, 12–18, 19–23, 24–30, 36–57, 36–71, 58–71, 76–79, 80–95, 81–95, all of them deriving from digestion within residues 1–95 of AMP deaminase, the fragments A-L-D-L-I-N-K and D-G-Y-L-F-Q-L-L-R were detected, corresponding to the rabbit HPRG fragments 16–22 and 26–34 respectively. An additional sequence G-E-V-L-P-L-P-E-A-N-F-P-S-F-X-L-R-H was found, where X can be serine, cysteine or some covalently modified amino acid. This sequence is similar to, but not identical with, residues 460–477 of the rabbit HPRG sequence (G-E-V-L-P-L-P-E-A-N-F-P-Q-L-L-R-N). Residues 472 and 473 (Q-L) in the published rabbit HPRG sequence are replaced by the sequence S-F that has already been reported for the corresponding residue of human and bovine HPRG [13]. In addition, residue 477 of the rabbit HPRG sequence (N) is replaced by H in the HPRG isoform identified in this study.

DISCUSSION

The only detailed analyses of the subunit structure of skeletal-muscle AMP deaminase reported in the literature, are those of Coffee and Kofke [10] and Boosman and Chilson [11], claiming on the basis of tryptic peptide maps that the enzyme is composed

of four identical or nearly identical 60 kDa (rat) or 69 kDa (chicken and rabbit) subunits. Our suggestion that the preparation of AMP deaminase that was analysed by these authors had undergone proteolysis [1] was confirmed from the sequence of skeletal-muscle AMP deaminase gene, which indicated that the rat as well as the human enzyme is composed of 85 kDa subunits [12]. By coupling this finding with the well-documented tetrameric nature of skeletal-muscle AMP deaminase, a 340 kDa molecular mass could be deduced for the whole enzyme. However, the data of the literature indicate that the molecular mass of the enzyme from various species ranges from 238 to 326 kDa [3–11]. These different reports have been explained by the inherent instability of the enzyme, undergoing fragmentation during the prolonged extraction procedure [1].

In a recent paper we reported that analysis by sedimentation–equilibrium techniques revealed an average molecular mass of 309 000 for the native rabbit enzyme, whereas after limited proteolysis with trypsin, which removes from the enzyme subunit the N-terminus up to residue 95 [2], AMP deaminase sedimented as a trimer of 222 000 Da [3]. Since the trypsinized enzyme is no longer sensitive towards stimulation by K^+ , a role for the fragment which is removed by proteolysis has been suggested in the maintenance of the tetrameric structure that is at the basis of the homotropic positive co-operativity shown by the enzyme at low K^+ concentrations [3]. The results of this paper, presenting evidence of heterogeneity in the subunit composition of rabbit skeletal-muscle AMP deaminase, suggest that it is necessary to propose a new model for the quaternary structure of the enzyme. Understanding how the different subunits of the enzyme interact and their functions will require further experiments to elucidate the structure and properties of the novel component of AMP deaminase. However, the present data establish a striking similarity between this peptide and HPRG, a plasma glycoprotein belonging to the cystatin superfamily [23]. First, amino acid analysis of the isolated peptide revealed a high content of both histidine and proline, which is the most salient feature of HPRG. Secondly, by N-terminal sequence analysis of freshly prepared AMP deaminase, a minor sequence was detected (L-T-P-T-D-X), corresponding to that of rabbit HPRG N-terminus [13]. Since this sequence was present in approximately equal amounts to the two minor sequences derived by proteolysis at positions 1 and 3 of the known subunit of AMP deaminase, it can be deduced that in comparison with HPRG the putative HPRG-like component of AMP deaminase contains an additional fragment with a blocked N-terminus, which is liberated by a proteolytic process during purification of the enzyme. This hypothesis is strengthened by the observation that a fragment with a blocked N-terminus found among those liberated by proteolysis with pepsin, of either whole AMP deaminase or the novel component of the enzyme, shows an amino acid composition quite different from that of the N-terminus of the known subunit of AMP deaminase. Thirdly, digestion with carboxypeptidase of native AMP deaminase failed to liberate stoichiometric amounts of any amino acid, whereas arginine was released by carboxypeptidase digestion of trypsinized AMP deaminase. The first observation may be explained by the presence in the native enzyme of C-terminal groups resistant to the exopeptidases, the known C-terminal sequences of skeletal-muscle AMP deaminase and HPRG being T-E and P-K respectively [12,13]. Concerning the presence of a C-terminal arginine in trypsin-cleaved AMP deaminase, it cannot be ascribed to the liberation of a C-terminal fragment from the known enzyme subunit, which is split by trypsin only at the N-terminus [2], but it may be accounted for by cleavage of the putative HPRG-like component of AMP deaminase, comprising a trypsin cleavage site corresponding to

Figure 6 Diagram of the effects of limited tryptic digestion on the molecular structure of rabbit skeletal-muscle AMP deaminase

(A) Schematic representation of the putative disulphide loop in the HPRG-like component. The thick arrow indicates the position where the tryptic cleavage liberates the N-terminal fragment that is additional with respect to HPRG. The trypsin cleavage sites corresponding to the major plasmin sites in rabbit HPRG are denoted by the two smaller arrows. The sequencing data refer to the HPRG-like peptide; numbers are those of the amino acid sequence of rabbit HPRG [13]. (B) Main peptides produced by trypsinization of whole AMP deaminase. Apparent molecular masses (mol. wt.) by SDS/PAGE (SDS-PAGE) are given on the right.

the major plasmin cleavage site (Arg-295) present in rabbit HPRG [13]. Fourth, fractionation by gel filtration on Sephadex G-50 of the peptide mixture obtained by treating AMP deaminase at pH 6.0 for 5 min with trypsin (150:1, w/w), yielded a first peak that was shown by SDS/PAGE analysis to contain four fragments with apparent molecular masses of 30, 40 (double band) and 70 kDa. Electrophoretic and sequencing analysis of the 30 kDa band revealed the sequence H-R-P-F-G-T-X-E-T-H-R-F, corresponding to the rabbit HPRG fragment beginning at His-296 [13], whereas the 40 kDa and 70 kDa bands gave this sequence together with a second sequence (L-T-P-T-D-X-K-T-T-K-P-L-A-E) corresponding to that of rabbit HPRG N-terminus [13].

A plausible interpretation of these data can be given taking into account the domain structure of rabbit HPRG [13], as well as the disulphide-linked fragments, which were shown to be produced by plasmin cleavage of that protein at Arg-295 and Lys-413; it has been shown that plasmin liberates from rabbit HPRG a 30 kDa fragment encompassing the histidine-proline-rich region (residues 296–413) and a 40 kDa fragment having the same N-terminal sequence as the intact protein [13]. Furthermore, the observation that CNBr-treated bovine HPRG migrated on SDS/PAGE as intact HPRG, giving a 75 kDa peptide, was explained as being due to a disulphide bridge connecting the N-terminal and C-terminal halves of the degraded protein [24]. On the basis of the homology with the bovine protein, the presence was inferred, also in rabbit HPRG, of a disulphide bridge connecting the N-terminal domain to the C-terminal domain (between Cys-6 and Cys-497).

With the assumption that this disulphide bridge is also a feature of the HPRG-like component of AMP deaminase, a schematic diagram of this peptide can be given (Figure 6A) that permits localization of the fragments observed in our SDS/PAGE analysis. Due to the disulphide bridge, the molecule cleaved by trypsin inside the disulphide loop at residue 295 behaves as a 70 kDa fragment, showing, in comparison with the intact protein, only removal of an approx. 5 kDa N-terminal fragment. The 40 kDa fragments, showing the same two N-terminal sequences of the 70 kDa fragment, probably derive from the reduction of the disulphide bridge, causing the separation of two peptides of apparent similar size (corresponding to the fragments 1–295 and 296–518 of rabbit HPRG). The single sequence shown by the 30 kDa band can be identified with the fragment corresponding to the histidine-proline-rich region of rabbit HPRG (residues 296–413), liberated by cleavage of the protein at two bonds inside the disulphide loop (residues 295 and 413). Our finding that arginine is released as a consequence of the digestion of trypsinized AMP deaminase by carboxypeptidases A and B strengthens the suggestion of correspondence between one of the trypsin cleavage sites in AMP deaminase and the plasmin cleavage site in rabbit HPRG at Arg-295 [13].

The observation that all rabbit HPRG domains migrate in SDS/PAGE at higher apparent molecular masses than those deduced from their sequences, has been explained by the presence in the HPRG molecule of six putative glycosylation sites [13]. As far as the 30 kDa fragment is concerned, the discrepancy due to the presence of oligosaccharides linked to Asn-302 may be enhanced by the high proline content of this domain. It also seems likely that the heterogeneity with respect to carbohydrate attachment should be responsible for the double band appearance in SDS/PAGE of the 40 kDa fragment. The effects of limited tryptic digestion of whole rabbit skeletal-muscle AMP deaminase are summarized in Figure 6(B).

In conclusion, the present data give evidence that rabbit skeletal-muscle AMP deaminase preparation contains two different protein species, one of them being the known catalytic subunit and the other showing similarity with plasma HPRG.

The possibility that the second component could be plasma HPRG co-purifying with the enzyme can be excluded on the basis of the sequence results obtained for the fragments liberated by a more extensive trypsinization of the AMP deaminase preparation. This analysis reveals some degree of divergence between the novel component and rabbit plasma HPRG in the region containing residues 472–477. These results indicate the presence of a novel peptide within the AMP deaminase complex and identify this additional peptide as a muscle-specific form of HPRG.

In comparison with mature HPRG, the AMP-deaminase-associated variant probably contains a unique N-terminal extension, but it diverges from rabbit plasma HPRG only at four amino acid residues among the 60 sequenced up to now. These observations suggest that a possible mechanism for the production of the HPRG-like peptide is the alternative splicing of the HPRG primary transcript producing an mRNA encoding a variant with a unique N-terminus spliced onto the mature HPRG polypeptide.

The present data do not permit us to exclude the possibility that the HPRG-like peptide may bind to AMP deaminase in the soluble extract without being part of its tetrameric structure. However, immunohistochemistry performed on human skeletal-muscle specimens using a rabbit anti-HPRG antibody detected a clear positive reaction at the level of the myofibril (A. Sabbatini, M. Ranieri-Raggi, A. J. G. Moir and A. Raggi, unpublished work). Since it has been reported that AMP deaminase is bound

to the myofibril in the A band [25], our observation suggests that the HPRG-like peptide could participate in the structure of native AMP deaminase. Although the physiological role of plasma HPRG remains unclear, it has been suggested that a function of this protein may be to bring two or more ligands together [13]. Therefore another possible explanation for the co-localization of AMP deaminase and the HPRG variant in skeletal muscle could be the participation of the novel peptide in the well-known protein-protein interactions of the enzyme with both myosin and titin [26,27].

One of the most studied interactions of HPRG is that with heparin, a mucopolysaccharide that exerts an anticoagulant effect. HPRG interferes with the heparin-promoted formation of the antithrombin-thrombin complex [28,29]. Interestingly, the circulatory clearance of chicken-muscle AMP deaminase is inhibited by heparin, suggesting that the observed rapid disappearance of AMP deaminase from the circulation may be due to the binding and internalization of the enzyme by the hepatocytes through the interaction with cell-surface heparan sulphate [30,31]. Furthermore, we have observed that rabbit-muscle AMP deaminase is strongly inhibited by heparin (M. Ranieri-Raggi, F. Ronca, A. Sabbatini and A. Raggi, unpublished work), which is in agreement with the observation that the chicken enzyme has a high affinity for sulphated polysaccharides [31]. The existence of a HPRG-like component in the AMP deaminase structure, as evidenced by the present paper, gives a possible molecular basis of the enzyme interaction with heparin.

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REFERENCES

- Ranieri-Raggi, M. and Raggi, A. (1980) *Biochem. J.* **189**, 367–368
- Ronca, F., Ranieri-Raggi, M., Brown, P. E., Moir, A. J. G. and Raggi, A. (1994) *Biochim. Biophys. Acta* **1209**, 123–129
- Ranieri-Raggi, M. and Raggi, A. (1990) *Biochem. J.* **272**, 755–759
- Lee, Y. P. (1957) *J. Biol. Chem.* **227**, 993–998
- Currie, R. D. and Webster, H. L. (1962) *Biochim. Biophys. Acta* **64**, 30–40
- Wolfenden, R., Tomozawa, Y. and Bamman, B. (1968) *Biochemistry* **7**, 3965–3970
- Ronca-Testoni, S., Ranieri, M., Raggi, A. and Ronca, G. (1970) *Ital. J. Biochem.* **19**, 262–276
- Zielke, C. L. and Suelter, C. H. (1971) *J. Biol. Chem.* **246**, 2179–2186
- Ashman, L. K. and Atwell, J. L. (1972) *Biochim. Biophys. Acta* **258**, 618–625
- Coffee, C. J. and Kofke, W. A. (1975) *J. Biol. Chem.* **250**, 6653–6658
- Boosman, A. and Chilson, O. P. (1976) *J. Biol. Chem.* **251**, 1847–1852
- Sabina, R. L., Morisaki, T., Clarke, P. R. H., Eddy, R., Shows, T. B., Morton, C. C. and Holmes, E. W. (1990) *J. Biol. Chem.* **265**, 9423–9433
- Borza, D. B., Tatum, F. M. and Morgan, W. T. (1996) *Biochemistry* **35**, 1925–1934
- Ranieri-Raggi, M. and Raggi, A. (1979) *FEBS Lett.* **102**, 59–63
- Ranieri-Raggi, M., Moir, A. J. G. and Raggi, A. (1985) *Biochim. Biophys. Acta* **827**, 93–100
- Zielke, C. L. and Suelter, C. H. (1969) *Fed. Proc. Am. Soc. Exp. Biol.* **28**, 728
- Panyim, S. and Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337–346
- LeGendre, N. and Matsudaira, P. (1989) in *A Practical Guide to Protein and Peptide Purification for Microsequencing* (Matsudaira, P., ed.), Academic Press, New York
- Moore, S. and Stein, W. H. (1963) *Methods Enzymol.* **6**, 819–831
- O'Driscoll, D., Roring, N. P. and Ross, C. A. (1976) *Biochem. Soc. Trans.* **4**, 928–930
- Edelhoch, H. (1967) *Biochemistry* **6**, 1948–1954
- Wilkinson, J. M. (1974) *Biochim. Biophys. Acta* **359**, 379–388
- Koide, T. and Odani, S. (1987) *FEBS Lett.* **216**, 17–21
- Soerensen, C. B., Krogh-Pedersen, H. and Petersen, T. E. (1993) *FEBS Lett.* **328**, 285–290
- Ashby, B., Frieden, C. and Bischoff, R. (1979) *J. Cell Biol.* **81**, 361–373
- Ashby, B. and Frieden, C. (1977) *J. Biol. Chem.* **252**, 1869–1872
- Soteriou, A., Gamage, M. and Trinick, J. (1993) *J. Cell Sci.* **104**, 119–123
- Lijnen, H. R., Hoylaerts, M. and Collen, D. (1983) *J. Biol. Chem.* **258**, 3803–3808
- Peterson, C. B., Morgan, W. T. and Blackburn, M. N. (1987) *J. Biol. Chem.* **262**, 7567–7574
- Husic, D. H. and Suelter, C. H. (1984) *J. Biol. Chem.* **259**, 4359–4364
- Husic, D. H. and Suelter, C. H. (1984) *J. Biol. Chem.* **259**, 4365–4371