Regulation of skeletal-muscle AMP deaminase: involvement of histidine residues in the pH-dependent inhibition of the rabbit enzyme by ATP

Maria RANIERI-RAGGI, Francesca RONCA, Antonietta SABBATINI and Antonio RAGGI*

Istituto di Chimica Biologica dell'Università di Pisa, Via Roma 55, 56100 Pisa, Italy

Reaction of rabbit skeletal-muscle AMP deaminase with a low molar excess of diethyl pyrocarbonate results in conversion of the enzyme into a species with one or two carbethoxylated histidine residues per subunit that retains sensitivity to ATP at pH 7.1 but, unlike the native enzyme, it is not sensitive to regulation by ATP at pH 6.5. This effect mimics that exerted on the enzyme by limited proteolysis with trypsin, which removes the 95-residue N-terminal region from the 80 kDa enzyme subunit. These observations suggest involvement of some histidine residues localized in the region HHMQAHILH (residues

51–60) in the regulatory mechanism which stabilizes the binding of ATP to its inhibitory site at acidic pH. Carbethoxylation of two histidine residues per subunit abolishes the inhibition by ATP of the proteolysed enzyme at pH 7.1, suggesting the obligatory participation of a second class of histidine residues, localized in the 70 kDa subunit core, in the mechanism of the pH-dependent inhibition of the enzyme by ATP. At a slightly acidic pH, these histidine residues would be positively charged, resulting in a desensitized form of the enzyme similar to that obtained with the carbethoxylation reaction.

INTRODUCTION

AMP deaminase (AMP aminohydrolase, EC 3.5.4.6), which catalyses the hydrolytic deamination of AMP to IMP and NH_3 , is widely distributed in animal tissues, but its level of activity in skeletal muscle is particularly high when compared with that found in all other tissues, including heart and smooth muscle [1]. The function of AMP deaminase in muscle operation is not clearly defined. However, on the basis of the considerably high specific activity of the enzyme in skeletal muscle, AMP deaminase in this tissue is very likely to play a significant role in regulating the relative concentration of adenine nucleotides.

For a long time it has been believed that the regulatory properties of skeletal-muscle AMP deaminase were similar to those of the enzyme from other sources in showing inhibition by guanine nucleotides and activation by adenine nucleotides [2]. Our studies on the regulatory properties of skeletal-muscle AMP deaminase from five different species at a KCl concentration close to that found in the muscle showed a constant inhibitory effect by ATP as well as by GTP, which was maximal at alkaline pH; since ADP was the most efficient metabolite in counteracting this inhibition, a decrease in the ATP/ADP ratio, together with a decrease in the tissue pH, was suggested to be the stimulus for the activation of AMP deaminase in periods of intense muscular activity [3].

Previous investigations in our laboratory have demonstrated that, in rat skeletal-muscle AMP deaminase, the sensitivity to allosteric inhibition by NTPs can be influenced by modifying either cysteine or lysine residues. Partial desensitization towards ATP and GTP was observed in the enzyme after modification of the thiol groups accessible to 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in the native protein [4]. The specific modification of the NTP-binding sites was shown to be the result of the modification of six or seven lysine residues by pyridoxal phosphate and NaBH_4 , which occurs without an appreciable decrease in the

enzyme V_{max} [5]. The involvement of lysine residues in the regulatory sites of the enzyme was confirmed by means of affinity labelling with periodate-oxidized ATP and GTP, which showed that 3.2 and 1.9 mol of these reagents respectively bind per mol of enzyme [6,7]. In two recent papers we have shown that limited proteolysis with trypsin of rabbit skeletal-muscle AMP deaminase removes a 10 kDa N-terminal fragment, desensitizing the enzyme to the inhibition by ATP at pH 6.5, but not at pH 7.1, while the inhibition by GTP is by no means affected by trypsin treatment [8,9].

The above observations stimulated our interest in finding a reagent which might selectively modify the binding site of either nucleotide. As shown by the present results, this was achieved by allowing rabbit skeletal-muscle AMP deaminase to react with diethyl pyrocarbonate (DEP), a fairly specific reagent for histidine residues. The results indicate that at least two classes of histidine residues are involved in the regulation of the enzyme, strongly suggesting the specific participation of histidine residues in the mechanism responsible for the pH-dependent allosteric inhibition of AMP deaminase by ATP.

MATERIALS AND METHODS

Enzyme

AMP deaminase was prepared as described previously [10] from fresh muscle dissected from the back and hind leg of rabbits. Limited proteolysis of AMP deaminase with trypsin and separation of the proteolysed protein from trypsin and by-products were carried out as described in [11]. Specific-activity values of 1100 and 900 μmol of AMP deaminated/min per mg of protein were determined when native and trypsin-treated AMP deaminases respectively were assayed in 50 mM imidazole/HCl (pH 6.5)/60 mM KCl/2 mM AMP at 20 °C.

The AMP deaminase activity was determined spectrophotometrically at 265 or 285 nm; $\Delta\epsilon_{\text{mM}}$ values of 8.86 and 0.23

were used at 265 and 285 nm respectively to calculate the amount (in μmol) of substrate deaminated [12].

Protein concentration was calculated from the A_{280} by using an $A_{280}^{1\%,1\text{cm}}$ value of 9.1 [13]. The molecular masses of the native and trypsin-treated enzymes were taken as 320 and 280 kDa respectively, assuming they contained four identical subunits of molecular masses 80 and 70 kDa respectively [9]. Spectrophotometric determinations were carried out in a Shimadzu UV-260 spectrophotometer.

Treatment with DEP

Chemical modification of AMP deaminase by DEP was carried out at 20 °C in 10 mM acetate buffer, pH 6.0, containing 1 M KCl. The reaction was initiated by the addition of 2–10 μl of a diluted ethanolic solution of DEP to 0.5–1.0 ml of the enzyme solution. At intervals, samples of the incubation mixture were withdrawn and assayed for catalytic activity, using a 700-fold dilution in 50 mM imidazole/HCl (pH 6.5 or 7.1), 0.1 mM AMP and 100 mM KCl. The concentration of DEP was determined from the increase in A_{230} when an aliquot of the DEP solution was added to 2 ml of 50 mM imidazole/HCl, pH 7.0, using an absorption coefficient for *N*-carbethoxyimidazole of $3000 \text{ M}^{-1}\cdot\text{cm}^{-1}$ [14].

The amount of *N*-carbethoxyhistidine residues in AMP deaminase was calculated from the difference in A_{242} between equal concentrations of DEP-treated enzyme and native enzyme, using the absorption coefficient of $3200 \text{ M}^{-1}\cdot\text{cm}^{-1}$ determined for *N*-carbethoxyhistidine at pH 6.0 [15].

After about 30 min incubation of AMP deaminase with DEP, no further variation in the absorbance of the reaction mixture was observed, in keeping with the reported 15 min half-life of DEP, due to the hydrolysis of the reagent in aqueous solutions at pH 6.0 [16].

Reversal of carboxylation with neutral hydroxylamine

A 0.1 ml sample of DEP-treated AMP deaminase was diluted in 0.2 ml of 1 M KCl/0.5 M hydroxylamine hydrochloride solution, adjusted to pH 7.0 by the addition of NaOH. At various times during incubation, samples were removed and tested by using a 400-fold dilution in the described assay mixtures.

Determination of SH groups

Titration of AMP deaminase thiol groups with DTNB were carried out at room temperature as described in [17]. Under non-denaturing conditions, the reaction mixture contained 0.4 mM DTNB, 50 mM Tris/HCl, pH 8.0, 0.9 M KCl and 0.4 mg/ml AMP deaminase. Thiol groups were also titrated in the denatured enzymes (0.15 mg/ml) using a reaction mixture containing 6 M urea. The enzymes previously treated with DEP were chromatographed on a column (0.6 cm $\times$ 12 cm) of Sephadex G-25 equilibrated and eluted with the buffer solution used for the thiol titration. Uptake of DTNB was calculated by using an absorption coefficient (ϵ_{412}) of $13600 \text{ M}^{-1}\cdot\text{cm}^{-1}$ [17] for the released 2-nitro-5-thiobenzoate.

Kinetic studies

The effect of histidine modification by DEP on the regulatory properties of AMP deaminase was studied by comparing the kinetic behaviour of the trypsin-treated enzyme with different extents of histidine modification by DEP with that of the untreated proteolysed enzyme. With the purpose of obtaining stable carboxylated enzyme species, at the end of the in-

cubation of trypsin-treated AMP deaminase with DEP the reaction mixture (0.5 ml) was applied to a column (0.6 cm $\times$ 12 cm) of Sephadex G-25 equilibrated and eluted with 1 M KCl, pH 7.0, to afford a separation of the carboxylated protein from ethanol and unchanged DEP.

Materials

Trypsin, DEP, DTNB and nucleotides were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephadex G-25 and G-50 were from Pharmacia; the other reagents used were of analytical grade.

RESULTS

Reversible desensitization of AMP deaminase to inhibition by NTPs

Figure 1(a) shows that 40 min incubation of rabbit skeletal-muscle AMP deaminase with DEP at pH 6.0 (molar ratio DEP/enzyme subunit, 25:1) does not appreciably affect the enzyme activity at 0.1 mM AMP, but results in a progressive loss of enzyme sensitivity towards inhibition by ATP at either pH 6.5 or 7.1. At the end of the treatment the activities in presence and in absence of ATP are thus indistinguishable. The same treatment desensitizes only partially AMP deaminase towards the inhibition by GTP.

Figure 1(b) shows that incubation of the DEP-treated enzyme with neutral hydroxylamine results in an almost complete restoration of ATP as well as GTP inhibition within 30 min. Incubation of AMP deaminase at pH 6.0 without DEP, over the experimental period, had no effect on the enzyme activity assayed in the absence or presence of each nucleotide (results not shown).

The different effect of the treatment with DEP on the modulation of AMP deaminase by the two NTPs suggests the existence of separate sites for ATP and GTP. This hypothesis is further supported by the different protective effects exerted by the two nucleotides on the DEP-induced desensitization of AMP deaminase (results not shown). We observed that, during the first 10 min incubation, the presence of 200 μM GTP did not modify the extent of desensitization of AMP deaminase by DEP, while the presence of 200 μM ATP reduced the loss of inhibition by ATP as well as by GTP. During the subsequent phase of the treatment, GTP, but not ATP, exerted protection against the DEP effect. At the end of the reaction in the presence of either nucleotide a similar reduction of the desensitization phenomenon was observed.

Nature of the groups reactive with DEP

DEP is expected to react fairly specifically with histidine residues in proteins at pH 6.0 to yield an *N*-carbethoxyhistidine derivative. However, modification by DEP of thiol groups and arginine, lysine and tyrosine residues has been reported [18]. Evidence that DEP reacts with histidine residues of AMP deaminase comes from the difference-absorption-spectrum analysis. When compared with the native enzyme, the DEP-treated enzyme shows an increase in absorption of the protein at about 245 nm (Figure 2), a feature of the reaction of DEP with histidine, indicating formation of *N*-carbethoxyhistidine residues [15]. The increase of A_{245} was abolished by the reaction of the modified protein with hydroxylamine (results not shown). Neutral hydroxylamine is known to remove the carboxy groups from either histidine or tyrosine modified residues [18]. However, *O*-carboxylation of tyrosine residues, which would cause a decreased A_{278} [19], can be ruled out in DEP-treated AMP deaminase, since the

Figure 1 Reversible desensitization of AMP deaminase to inhibition by NTPs

(a) The enzyme (2.5 μM) was incubated with 250 μM DEP as described in the Materials and methods section and assayed at the indicated times for the catalytic activity at 0.1 mM AMP in the absence (▲, △) or in the presence of 10 μM ATP (●, ○) or 10 μM GTP (■, □). (b) After 40 min, the incubation mixture was diluted 3-fold in 0.3 M hydroxylamine/HCl, pH 7.0, and tested for activity as in (a). Open and closed symbols refer to determinations at pH 6.5 or pH 7.1 respectively. The 100% value is assigned to the enzyme activity in the absence of effectors at zero time of incubation.

absorption at 278 nm of the modified enzyme turned out to be identical with that of the native enzyme.

Since it has been reported that modification with DTNB of about three SH groups per subunit of rat skeletal-muscle AMP deaminase partially desensitizes the enzyme to the effects of NTPs [4], the possible modification of thiol groups by DEP was

Figure 2 Spectra of native and carbethoxylated AMP deaminases

Individual spectra (—) of AMP deaminase (3.4 μM in 1 M KCl), native (lowest curve) or carbethoxylated (upper curves), were recorded with 1 M KCl, pH 7.0, as blank. The broken curves show the difference spectra obtained when either modified AMP deaminase sample was compared with the native enzyme. Carbethoxylated enzymes contained 2.7 mol (---) and 1.8 mol (---) of modified histidine per mol of enzyme subunit.

Table 1 Effect of chemical modification of AMP deaminase by DEP on the reaction of the enzyme with DTNB

Results are means ± S.D. for three determinations, except where indicated by an asterisk, where the result is from six determinations.

Subunit and treatment	Thiol groups titrated per subunit	
	Non-denatured	Denatured
80 kDa subunit		
Untreated	2.5 ± 0.3*	7.3 ± 0.2
With 4.7 carbethoxyhistidine residues	2.1 ± 0.2	7.3 ± 0.3
70 kDa trypsin-treated subunit		
Untreated	1.3 ± 0.2	5.6 ± 0.1
With 3.4 carbethoxyhistidine residues	1.4 ± 0.1	5.4 ± 0.3

tested by comparing titrations with Ellman's reagent of free thiols in untreated and DEP-treated AMP deaminases. Table 1 reports the results obtained titrating both whole AMP deaminase and the product of limited proteolysis of the enzyme by trypsin, which removes the 95-residue N-terminal fragment from the 80 kDa subunit [9]. The first and second columns of Table 1 show the number of thiol groups per subunit titrated with DTNB in the absence or presence of 6 M urea respectively. The average values obtained in the two conditions with untreated rabbit skeletal-muscle AMP deaminase, corresponding to 10 and 29 thiol groups per enzyme molecule, are somewhat lower than those titrated in the rat enzyme, namely 12.4 and 30.6 respectively [20]. The significant decrease in the titratable thiol groups observed in the enzyme as consequence of limited proteolysis (Table 1) can be well explained by the observation that the N-terminal fragment removed by trypsin from skeletal-muscle AMP

Figure 3 Correlation between the inhibition of native and trypsin-treated AMP deaminases by NTPs and the number of modified histidine residues

After 40 min reaction of native (a) or trypsin-treated (b) AMP deaminase (3.4 μ M) with different DEP concentrations (20–100 μ M), the *N*-carboxyhistidine content was determined from the A_{242} of the incubation mixture, and AMP deaminase activity was assayed as described in the Materials and methods section in the absence or in the presence of 10 μ M ATP or 10 μ M GTP, at pH 6.5/60 mM KCl (open symbols) or at pH 7.1/150 mM KCl (closed symbols). $\circ$, $\bullet$, Residual inhibition by ATP; $\square$, $\blacksquare$, residual inhibition by GTP. The 100% value is assigned to the inhibition of the enzyme by either nucleotide at zero time of incubation.

deaminase contains one cysteine residue at position 47 [9]. Table 1 shows that titrations of thiol groups in native as well as in trypsin-treated AMP deaminase at the indicated extent of histidine carbethoxylation give values very similar to those obtained with the corresponding unmodified enzymes. These data indicate that the modification of AMP deaminase by DEP does not involve thiol groups.

Involvement of histidine residues in the inhibition of AMP deaminase by ATP and GTP

In order to correlate the extent of the desensitization of AMP deaminase to inhibition by ATP and GTP with the number of histidine residues modified by DEP, the enzyme was incubated with different stoichiometric amounts of DEP for 40 min. At the end of this treatment, the inhibition of the enzyme by ATP and GTP was determined at two different pH values (pH 6.5 and pH 7.1), and the recorded increase in absorption at 242 nm of the incubation mixture was taken as a measure of the number of carbethoxylated histidine residues.

A linear correlation was observed between the extent of histidine carbethoxylation and the concentration of DEP in the reaction mixture up to a ratio of 5 mol of DEP/mol of AMP deaminase subunit when the modification of about three histidine residues/subunit was determined. With a larger molar excess of the reagent the progression of the acylation reaction decreased, indicating the involvement of a class of histidine residues with a lower reactivity.

Although a similar time course for the loss of inhibition of AMP deaminase by ATP at either pH 6.5 or 7.1 is observed during incubation of the enzyme subunit with a 25 molar excess of DEP (Figure 1), at a lower extent of histidine residue modification different consequences on the regulatory properties of the enzyme at either pH value are observed. This is shown in Figure 3(a), where the experimental data of histidine modification are plotted on a semilogarithmic scale against the residual inhibition by the allosteric effectors. The progression of desensitization towards inhibition by ATP at pH 6.5 is linear and

almost complete when one or two histidine residues per subunit have been modified. By contrast, at pH 7.1, the correlation is not linear, indicating that, among the most reactive histidine residues, at least two classes can be distinguished on the basis of the consequences of their modification on the enzyme kinetic properties.

A similar progression of desensitization of AMP deaminase towards GTP inhibition is observed at either pH 6.5 or 7.1. In both cases, an initial increase in absorbance at 242 nm is obtained with no appreciable concomitant loss of inhibition by GTP, confirming that the partial removal of the modulation of the enzyme by GTP is due to modification of histidine residues less reactive than those involved in the binding of ATP.

The above results show that the DEP-induced modification of one or two histidine residues per subunit exerts an effect similar to that of limited proteolysis on the modulation of AMP deaminase by ATP. As a matter of fact, trypsin removes the N-terminal 10 kDa fragment from rabbit skeletal-muscle AMP deaminase, desensitizing the enzyme towards ATP inhibition at pH 6.5, but not at pH 7.1 [8]. This observation suggests that trypsin removes from AMP deaminase a regulatory domain in which the most reactive histidine residues can be localized. This hypothesis is strengthened by the data of Figure 3(b), showing that the number of histidine residues per subunit that need to be modified in order to reach an almost complete desensitization towards ATP at pH 7.1 are reduced from about four in the native enzyme to about two in the proteolysed enzyme. Furthermore, differently to what was observed with the native enzyme, a linear correlation is observed between the loss of inhibition by ATP and the number of histidine residues modified.

The effect of the reaction with DEP on the inhibition of trypsin-treated AMP deaminase by GTP is not pH-dependent, a finding similar to what was observed with the native enzyme. Even in this case, the progression of the desensitization phenomenon is not linear, an initial reaction of about one histidine residue occurring with no significant change of the activity in the presence of GTP. However, the modification of one or two additional histidine residues induces a more drastic loss of inhibition by GTP in the proteolysed enzyme compared with what was observed with the native enzyme.

Difference-absorption-spectrum analysis (results not shown) showed that treatment of trypsin-treated AMP deaminase with DEP up to about three carbethoxylated histidine residues per subunit caused an increase in absorption of the protein at about 245 nm with no apparent decrease of the absorption at 278 nm, a finding similar to what was observed with the native enzyme. At higher extents of modification, quantification of histidine-residue modification by increase of absorption at 242 nm was complicated by an absorption decrease which showed a maximum below 240 nm. This phenomenon, coupled to the slight decrease of absorption at 278 nm observed under these conditions, suggests the reaction of DEP with tyrosine residues [19].

Effect of carbethoxylation on the regulatory properties of trypsin-treated AMP deaminase

The finding that more clear-cut consequences in the regulatory properties of AMP deaminase are induced by DEP in the trypsin-treated enzyme compared with the observation for the native enzyme prompted us to study the kinetic behaviour of the proteolysed enzyme with various extents of histidine modification.

As previously reported, proteolysed rabbit skeletal-muscle AMP deaminase over the pH range 6.5–7.1 exhibits hyperbolic

Figure 4 Effect of NTPs on trypsin-treated AMP deaminase with various extents of histidine carbethoxylation

The activity of trypsin-treated AMP deaminase before (○, ●) and after modification by DEP of two (△, ▲) or 3.5 (□, ■) histidine residues per subunit was assayed at 0.1 mM AMP in 50 mM imidazole/HCl containing 60 mM KCl at pH 6.5 (open symbols) or 100 mM KCl at pH 7.1 (closed symbols) and the indicated ATP (a) or GTP (b) concentrations. The 100% value is assigned to the activity of each AMP deaminase in the absence of effectors.

kinetics. On this enzyme ATP exerts a highly pH-dependent inhibitory effect which is absent at acid pH values and appears at pH 7.1, inducing sigmoid kinetics in the enzyme [8]. The results shown in Figure 4(a) demonstrate that the inhibition exerted by ATP on the proteolysed AMP deaminase at pH 7.1, which is very similar to that observed for the native enzyme (results not shown), is completely removed when two histidine residues per subunit are modified by DEP. The proteolysed enzyme with 3.5 carbethoxylated histidine residues per subunit is slightly stimulated by ATP at pH 6.5 as well as at pH 7.1.

GTP exerts on the proteolysed AMP deaminase, at pH 6.5 as well as at pH 7.1, inhibitory effects (Figure 4b) that are very

Figure 5 Effect of histidine carbethoxylation on the modulation of trypsin-treated AMP deaminase by increasing concentrations of ATP and ADP

The activity of trypsin-treated AMP deaminase before (a) and after (b) modification by DEP of two histidine residues per subunit was assayed in 50 mM imidazole/HCl (pH 7.1)/0.1 mM AMP/100 mM KCl and the indicated ADP (○, ●) or ATP (△, ▲) concentrations, in the absence (open symbols) or presence (closed symbols) of 2.5 μM GTP.

similar to those observed with the native enzyme (results not shown). The effect of GTP at both pH values is partially and almost totally removed by carbethoxylation of two and 3.5 histidine residues per subunit respectively.

The substrate-velocity data obtained with the DEP-treated proteolysed AMP deaminases at pH 7.1 in the presence of 10 μM ATP or 2.5 μM GTP and at pH 6.5 in the presence of 2.5 μM GTP were compared with that of the unmodified proteolysed enzyme (results not shown). The ATP-induced sigmoidicity of

the substrate saturation curve of the trypsin-treated enzyme at pH 7.1 [Hill coefficient (h) = 2.1] turned out to be clearly decreased as an effect of carbethoxylation of either two or 3.5 histidine residues per subunit (h = 1.4 and 1.3 respectively), the slope of the Hill plots being about the same value (h = 1.3) observed for either treated or untreated enzyme in the absence of ATP.

Accordingly to what was previously reported [8], unlike ATP, the effect of GTP was to induce sigmoid kinetics in the proteolysed enzyme at pH 6.5 as well as at pH 7.1 (h = 2.5 and 2.9 respectively). The carbethoxylation of two histidine residues per subunit decreased, but did not remove the positive-co-operativity behaviour observed in the presence of GTP (h = 1.8 at both pH values), whereas almost superimposable substrate-saturation plots were obtained in the presence or absence of GTP, using the enzyme in which 3.5 histidine residues per subunit were modified. However, it should be pointed out that at this extent of modification the enzyme showed at pH 6.5 a kinetic behaviour which may be interpreted as being due to a negative-co-operativity effect (h = 0.7). This phenomenon was not observed at pH 7.1 (h = 1.2), and this suggests that more extensive DEP treatment involves residues which are essential to a proper conformation of the protein at acidic pH, allowing the substrate to yield a full saturation of the enzyme.

Removal by ATP and ADP of the inhibitory effect of GTP in trypsin-treated AMP deaminase

The above results show that carbethoxylation of two histidine residues per subunit abolishes the inhibition of the proteolysed AMP deaminase by ATP, but not by GTP, suggesting the existence of separate sites for the two nucleotides. It has been reported, however, that the inhibitory effect of GTP on AMP deaminase is released by increasing concentrations of either ADP or ATP [3,21]. In order to ascertain whether histidine carbethoxylation may also modify the capability of ATP to stimulate an inhibited form of the enzyme, we tested the effect of increasing ATP or ADP concentrations on the activity of the proteolysed enzyme in the absence and presence of GTP, before and after modification of two histidine residues per enzyme subunit.

Figure 5(a) shows that, at pH 7.1, ATP exerts a biphasic effect on the proteolysed enzyme; $< 30 \mu\text{M}$ ATP acts as an inhibitor, whereas $> 30 \mu\text{M}$ ATP reverses the inhibition. An increasing concentration of ATP also releases GTP inhibition. Similar influences of ATP are observed with the native enzyme (results not shown). Figure 5(b) shows that the histidine modification cancels the biphasic effect of ATP, but not its ability to remove the inhibition by GTP. Under these conditions, the K_A for ATP stimulation, calculated from a plot of $[\text{ATP}]/v$ against $[\text{ATP}]$, was found to be $3.6 \mu\text{M}$ compared with $40 \mu\text{M}$ calculated using the data of Figure 5(a) obtained with the unmodified proteolysed enzyme. After DEP treatment, the stimulation of the proteolysed enzyme by increasing ADP concentrations is not significantly different from that observed before the DEP treatment (Figures 5a and 5b), the K_A values being $18 \mu\text{M}$ and $10 \mu\text{M}$ respectively. However, similarly to what was observed with ATP, the ability of ADP to remove the inhibitory effect of GTP is enhanced by the DEP treatment, K_A values of $112 \mu\text{M}$ and $9.2 \mu\text{M}$ being calculated before and after histidine modification.

DISCUSSION

The literature suggests that GTP and ATP have a common binding site on the skeletal-muscle AMP deaminase molecule, since they show mutually inhibitory binding to the enzyme [3,21].

However, some differences were observed in the effect of these nucleotides. ATP exerts a biphasic effect on the enzyme at K^+ concentrations lower than optimal; up to $10 \mu\text{M}$, ATP acts as an inhibitor, whereas at higher concentrations it slightly activates the enzyme. GTP inhibits the enzyme in every case [7,22,23]. Furthermore, using equilibrium-binding studies, 3.8 binding sites were found in the rabbit enzyme for ATP with an apparent dissociation constant, K_d , of $21 \mu\text{M}$, but only 2.0 for GTP with a K_d of $2 \mu\text{M}$, and treatment of the enzyme with *p*-mercuribenzoate selectively abolished binding of GTP [22]. In agreement with these data we found marked quantitative differences in the binding of periodate-oxidized ATP and GTP to rat skeletal-muscle AMP deaminase. We found 3.2 binding sites for oxidized ATP, with a K_d of $86 \mu\text{M}$, but only 1.9 for oxidized GTP, with a K_d of $6.8 \mu\text{M}$ [6,7].

Skeletal-muscle AMP deaminase is a tetramer, the subunit molecular mass being about 80 kDa. Partial proteolysis by trypsin converts the AMP deaminase subunit into a stable product of approx. 70 kDa, removing the 95-residue-long N-terminus of the enzyme [9,10]. The fragment removed by trypsin seems to have a specific recognition function in the parent protein, because proteolysed AMP deaminase is inhibited by GTP to the same extent as the original enzyme and retains sensitivity to ATP at pH 7.1, but is no longer sensitive to regulation by ATP at pH 6.5 [8]. The trypsin-treated enzyme also shows a complete loss of sensitivity towards the activation by ADP at pH 6.5, exhibiting hyperbolic kinetics even at low K^+ concentration and in the absence of ADP [10].

The present results on the effects of histidine modification by DEP in native AMP deaminase show that, among the most reactive histidine residues, a class of one or two per subunit can be distinguished, since their modification causes a complete release of ATP inhibition at pH 6.5, but not at pH 7.1. Since this effect mimics the one exerted on the enzyme by limited proteolysis with trypsin, we can suggest the involvement of histidine residues localized within the 95-residue N-terminal fragment in the regulatory mechanism which stabilizes the binding of ATP to its inhibitory site at acidic pH. In trypsin-treated AMP deaminase, which is no longer inhibited by ATP at pH 6.5, the modification of about two histidine residues per subunit gives an almost complete desensitization towards the inhibition by ATP at pH 7.1. We can therefore conclude that a second class of histidine residues, responsible for the inhibitory function of ATP at pH 7.1, is localized in the 70 kDa core.

These data confirm the previous observations of a highly pH-dependent inhibitory effect by ATP of rabbit skeletal-muscle AMP deaminase that has suggested the distinction of this phenomenon from the inhibition of the enzyme by GTP [8]. This hypothesis is strengthened by the present data on the effects of histidine-residue carbethoxylation by DEP on the regulatory properties of trypsin-treated AMP deaminase. As a matter of fact, the modification of two histidine residues per subunit removes the inhibitory effect of ATP, while the effect of GTP is abolished only at a higher extent of protein modification which induces negative co-operativity in the enzyme kinetics at optimal pH 6.5 in the absence of effectors. Negative-co-operativity behaviour is elicited in rat skeletal-muscle AMP deaminase by dinitrophenylation or nitration of one or two tyrosine residues [24,25]. Further studies are necessary to establish whether the negative co-operativity, observed in the rabbit proteolysed enzyme after extensive treatment by DEP, is due to tyrosine-residue modification. However, the present data suggest that the observed desensitization towards GTP may be due to an alteration of the three-dimensional structure of the enzyme subunit rather than to a direct modification of the GTP-binding site.

It should be pointed out that the kinetic studies described in the present paper were purposely carried out using the trypsin-treated enzyme, since this form of AMP deaminase can be regarded as a homogeneous simpler species in which the influence of pH is isolated from that of K^+ [8]. Nevertheless, the present data can be compared with those obtained by Ashby and Frieden [21] using an AMP deaminase preparation from frozen rabbit muscle, as we have previously observed that on storage a cleavage of the enzyme subunit occurs to a product similar in size to that obtained on limited proteolysis of AMP deaminase with trypsin [26].

The data of Ashby and Frieden [21] were consistent with an enzyme containing an inhibitory site relatively specific for NTPs and an activating site which can bind NMPs, NDPs and NTPs. Our kinetic data confirm the existence of at least two regulatory sites for NDPs and NTPs, but give evidence of further differentiation in the specificity of both the inhibitory and activating sites. In addition to the above described distinction between the inhibitory action shown by ATP and GTP, the results described in the present paper indicate that the stimulation of the inhibited enzyme observed at high levels of ATP or GTP ($> 100 \mu\text{M}$) [21,22,27] differs from the activating effect of ADP. In the proteolysed enzyme, desensitized towards the inhibition by ATP as effect of the DEP treatment, the ability of this nucleotide to remove the inhibition by GTP is still present and appears with an apparently higher affinity compared with what is observed for the untreated enzyme. The observation that the K_A for stimulation by ADP of the enzyme in the presence of GTP is also lowered after DEP treatment may be interpreted in terms of an activating site which can bind both ADP and ATP, producing a conformational change which releases GTP inhibition by weakening the GTP binding to its inhibitory site. However, higher concentrations of ATP remove the inhibition exerted by GTP (as well as by low concentrations of ATP in the untreated enzyme), causing the enzyme activity to revert to the same limited level observed at pH 7.1 in the absence of inhibitors. By contrast, increasing concentrations of ADP provoke enzyme stimulation, either in the presence or absence of inhibitors, up to the maximum level exhibited under optimal assay conditions, i.e. pH 6.5. The existence in the enzyme subunit of a specific site to which NDPs bind, directly activating the enzyme, is further suggested by the observation that the stimulating effect of ADP observed in the absence of GTP is not significantly influenced by the DEP-induced modification.

The most important finding in the present study remains the obligatory participation of some histidine residues in the mechanism of the modulation of rabbit skeletal-muscle AMP deaminase by ATP, which permits one to envisage the molecular basis of the pH-dependent inhibition of the enzyme by this effector. As concerns the DEP-induced desensitization of native enzyme to ATP inhibition at pH 6.5, an essential role in the constitution of the ATP binding site of the histidine residues involved in this phenomenon can be excluded, since they can be localized within the 95-residue N-terminal fragment removed by trypsin without affecting the inhibition by ATP at pH 7.1. Our recent sequence data [9] permit one to localize these histidine residues in the 51–60-residue region (HHMQAHILH) of the AMP deaminase subunit. These histidine residues may act indirectly by inducing or stabilizing a conformational change in the enzyme which permits the binding of ATP at pH 6.5. This hypothesis is strengthened by the observation that, although the two classes of histidines responsible for the ATP sensitivity at pH 6.5 and 7.1 may be distinguished on the basis of their reactivity towards stoichiometric amounts of DEP, this distinction is no longer permitted when the enzyme is treated with

a large molar excess of the reagent. As a matter of fact, under these conditions the desensitization phenomenon occurs at the same rate at the two pH values. To explain the apparent discrepancy of these results, it can be hypothesized that the conformational change due to modification of the most reactive histidine residues may be counteracted by that induced by the carbethoxylation process occurring only at high reagent concentration and causing the enzyme to show a negative-cooperativity behaviour at pH 6.5 (see above).

The observation that desensitization towards ATP at pH 7.1 occurs in the trypsin-treated enzyme when about two histidine residues per subunit are altered suggests their localization in the 70 kDa subunit core. The localization of the ATP-binding site towards the C-terminus of AMP deaminase has been suggested from the observation that residues 717–731 of the yeast enzyme bind 8-azido-ATP [28]. Comparison of the C-terminal region of skeletal-muscle AMP deaminase with that of the yeast enzyme shows conservation of a long amino acid sequence (residues 656–691) containing the potential ATP-binding site [29]. In the flanking regions of this peptide segment, skeletal-muscle AMP deaminase contains the only two histidine residues present in the C-terminal 150-residue region (His-655 and His-694). A recent review showed the sequences similarities among the regulatory sites that bind ATP in various enzymes, but the data appeared to be insufficient to establish a reliable consensus sequence for the ATP-binding site when the nucleotide acts as inhibitor [30]. Therefore the involvement of His-655 and His-694 in the constitution of the ATP-binding site in skeletal-muscle AMP deaminase appears to be somewhat speculative. Nevertheless, we may hypothesize that uncharged histidine residues at basic pH would permit the binding of ATP to its regulatory site. At a slightly acidic pH, these histidine residues would be positively charged, resulting in a desensitized form of the enzyme similar to that obtained with the carbethoxylation reaction.

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