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# SPECIFIC MODIFICATION OF THE GTP BINDING SITES OF RAT 5'-ADENYLIC ACID AMINOHYDROLASE BY PERIODATE-OXIDIZED GTP

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## Summary

1. Rat skeletal muscle AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) can be inactivated by incubation with the periodate-oxidized analogue of the enzyme inhibitor GTP.

2. Nucleoside triphosphates and KCl at high concentrations protect against inactivation, while ADP has no effect.

3. The inactivation can be reversed by the addition of GTP and amino acids and made irreversible by reduction with $NaBH_4$. This indicates that, in the binding of the oxidized GTP to the enzyme, a Schiff base is formed between the aldehyde groups of the inhibitor and amino groups of the enzyme.

4. The kinetic properties of the reduced (oxidized GTP)-AMP deaminase derivative indicate that the loss of activity results from an increase in $K_m$ while no appreciable change in $V$ is observed; consequently, the enzyme shows positive homotropic cooperativity even in the presence of optimal KCl concentration.

5. Since the treated enzyme shows kinetic properties similar to those of the native enzyme in the presence of GTP, and since the loss of sensitivity to GTP is directly proportional to the degree of inactivation, it is concluded that the oxidized GTP specifically modifies the binding sites for GTP.

6. Binding of the radioactive oxidized GTP shows that two binding sites for this reagent exist in the AMP deaminase molecule.

## Introduction

AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) from rat skeletal muscle is inhibited by GTP. This inhibition, which is reversed by ADP and high salt concentrations, results in a decreased affinity for AMP and causes the hyperbolic substrate-velocity curve obtained at optimal $K^+$ concentrations to revert to the sigmoidal one shown by the native enzyme in the absence of salt [1,2]. Kinetic behaviour similar to that of the native AMP deaminase in the presence

of GTP is shown by the enzyme after modification of 6—7 lysines with pyridoxal-5′-*P* and $NaBH_4$ [3]. Since the sensitivity of the enzyme towards ATP and GTP is completely lost after this treatment, it was suggested that pyridoxal-5′-*P* binds at the binding sites of these nucleoside triphosphates.

From the literature, it would appear that GTP and ATP have a common binding site on the skeletal muscle AMP deaminase molecule, since both nucleotides inhibit the activity of the enzyme at $K^+$ concentrations higher than 0.1 M [4], and show mutually inhibitory binding to rabbit muscle AMP deaminase [5]. However, some differences were observed in the effect of these nucleotides. GTP, but not ATP, increases the rate of inactivation of rat muscle AMP deaminase by EDTA [6]. ATP exerts a biphasic effect on the enzyme at $K^+$ concentrations lower than optimal; up to 5 $\mu$M, ATP acts as an inhibitor while at higher concentrations it slightly activates the enzyme. GTP inhibits the enzyme in every case [2]. Furthermore, using equilibrium binding studies, 3.8 binding sites were found in the rabbit enzyme for ATP but only 2.0 for GTP, and treatment of the enzyme with *p*-mercuribenzoate selectively abolished binding of GTP [5].

The above observations stimulated our interest in finding a reagent which might modify the binding site of either nucleotide with greater specificity than pyridoxal-5′-*P*. Periodate oxidation of the vicinal 2′-3′ alcoholic groups has been a useful approach for the binding of nucleotides and nucleic acids to resins suitable for affinity chromatography [7,8]. Besides this function, periodate-oxidized nucleotides can be useful materials for the specific modification of binding sites for coenzymes and effectors, as recently shown by Rippa et al. [9], who used periodate-oxidized NADP to modify 6-phosphogluconate dehydrogenase. The data reported in this paper present evidence that the periodate oxidation product of GTP can be used for the specific modification of the GTP binding sites of rat muscle AMP deaminase.

## Materials and Methods

### *Enzyme*

Rat muscle AMP deaminase was purified as previously described [10], except that elution of the enzyme from cellulose phosphate was performed with 1 M KCl adjusted to pH 7.0 with 1 M $KH_2PO_4$ after a previous elution with 0.6 M KCl, pH 7.0, which yielded one small peak of lower specific AMP deaminase activity attributable to the isoenzyme present in red muscle [11].

The homogeneous enzyme showed a specific activity of 1200 $\mu$mol of AMP deaminated per min per mg of protein at 20° C in 50 mM imidazole · HCl (pH 6.5), 100 mM KCl and 2 mM substrate. Protein concentration was determined from the absorbance at 280 nm assuming an $E^{1\%}_{1cm}$ value of 9.8 [10]. The molecular weight of the enzyme was taken as 290000 [10]. Enzyme activity was measured spectrophotometrically at 265 nm or 285 nm and the amount of AMP deaminated was calculated using $\Delta E_{mM}$ values of 8.86 and 0.23 at 265 and 285 nm, respectively [1].

### *Inactivation-reactivation studies*

The periodate oxidation product of GTP was prepared as has been described

[8] and isolated by precipitation of the barium salt. The concentration of the oxidized GTP was calculated spectrophotometrically by using a molar extinction coefficient of 11800 at 260 nm, pH 7.0. Incubation of the enzyme with different concentrations of oxidized GTP was carried in Tris · HCl buffer pH 8.0, at 20° C. At intervals samples of the reaction mixture were removed and immediately tested for catalytic activity using a 200-fold dilution in 50 mM imidazole · HCl (pH 6.5), 100 mM KCl and 0.1 mM AMP (or 2 mM AMP when specified). Control samples were incubated in the absence of oxidized GTP. To prepare a stable (oxidized GTP) · enzyme complex the enzyme, inhibited by incubation with oxidized GTP, was reduced by the addition of 10 μl of a fresh aqueous solution of 1 M $NaBH_4$ to 0.5 ml of the incubation mixture. The reduction was allowed to proceed for 30 min at 0° C and the reaction mixture was then dialysed for 24 h against several changes of 0.8 M KCl/0.02 M phosphate buffer (pH 6.9) at 4° C. Control samples were treated as above but in the absence of oxidized GTP.

Reactivation of the enzyme inhibited by oxidized GTP was performed using an enzyme with 7% residual activity which was diluted 6 times in an incubation mixture containing valine, GTP, or valine and GTP, 0.6 μM treated enzyme, 50 mM imidazole · HCl (pH 7.1) and 0.5 M KCl. At intervals samples were diluted 200-fold into an assay mixture containing 50 mM imidazole · HCl (pH 6.5), 100 mM KCl, 0.1 mM AMP with or without 50 μM ADP.

*Analytical measurements*

Binding of the oxidized GTP to the enzyme was measured by using the radioactive reagent obtained by periodate oxidation of [$^3$H]GTP. The amount of oxidized GTP bound per mol of protein was calculated from the specific radioactivity of (oxidized GTP) · protein and the original oxidized GTP solution. Protein concentration was determined by the method described by Lowry et al. [12], using native AMP deaminase as standard.

Determination of radioactivity was carried out in a Packard model 2002 Tri-Carb liquid scintillation spectrometer in the solvent system described by Bray [13]. Spectrophotometric determinations were made in a Zeiss PMQ II spectrophotometer equipped with a GOERZ Servogor S recorder. AMP and ATP were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.; ADP and GTP from Boehringer und Soehne, GmbH, Mannheim, West Germany; [8-$^3$H]GTP from the Radiochemical Centre, Amersham, Bucks, U.K.; the other reagents were of analytical grade.

## Results

*Reversible inactivation of AMP deaminase by the periodate oxidation product of GTP*

When rat muscle AMP deaminase is incubated with a 33-fold molar excess of oxidized GTP at pH 8.0 a rapid inactivation of the enzyme is observed (Fig. 1A). This loss of activity is biphasic since the first phase of the reaction, reaching an 80% loss of activity, is followed by a reduction of the inactivation rate. Since subtraction of the slower rate from the experimental values of the first phase gives a straight line on the semi-log plot, the inhibition of AMP deamin-

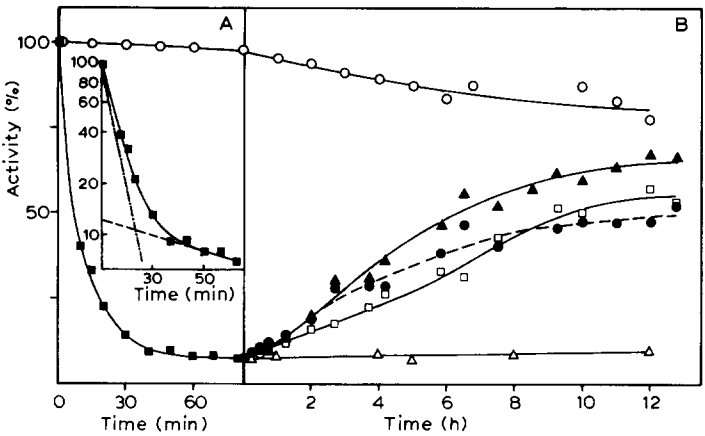



Fig. 1. Reversible inactivation of rat muscle AMP deaminase by periodate-oxidized GTP. A, enzyme residual activity as a function of time. The reaction mixture contained 30 mM Tris · HCl (pH 8.0), 0.9 M KCl, 2.4 μM AMP deaminase and 80 μM oxidized GTP added as the last reagent. Control samples (○) were incubated without oxidized GTP. Inset, semilog plot of the inactivation data. ———, observed residual activity at time indiacted; - - - - - -, extrapolation of slower rate to zero time; — · — ·, line obtained after subtraction of slower rate from observed activity. B, reactivation of periodate-oxidized GTP-treated enzyme by GTP and valine. AMP deaminase inactivated as in A was incubated at 20° C, pH 7.1, with 25 mM valine (□), or 0.2 mM GTP (●), or 25 mM valine plus 0.2 mM GTP (▲). △, (oxidized GTP) · enzyme incubated with 25 mM valine plus 0.2 mM GTP after $NaBH_4$ reduction. ○, untreated AMP deaminase diluted in the reactivation mixture at the end of incubation as in A.

ase by excess oxidized GTP may be described as the sum of two first-order reactions. A similar decrease in the inactivation rate at 20—25% residual activity was observed in the experiments carried out with 500-fold molar excess of oxidized GTP or using 0.9 M KCl/10 mM potassium phosphate buffer, pH 8.0.

The inhibitory effect of oxidized GTP is reversed by addition of GTP and amino acids to the treated enzyme (Fig. 1B). Initially, a faster rate of reactivation is observed with 0.2 mM GTP than with 25 mM valine, 27% and 19% restoration of the original activity, respectively, being observed after 3-h incubation. However, the extent of reactivation obtained after 12-h incubation is limited to 50% and 55% in the presence of GTP and valine respectively, but is raised to 67% when the inhibited enzyme is incubated with both valine and GTP. As is shown by Fig. 1B, the activity of the controls decay with incubation time to below 80% of the original activity. It can also be seen from Fig. 1B that when the (oxidized GTP) · enzyme complex stabilized by $NaBH_4$ is incubated with GTP and valine, there is no appreciable increase in enzyme activity.

The values reported in Fig. 1B were obtained by assaying enzyme activity in the presence of 50 μM ADP to remove the inhibitory effect exerted by GTP [10]. During the first 15 min of reactivation the enzyme activities assayed in the presence and absence of ADP were not appreciably different; subsequently, the activities in the absence of ADP were lower, 46%, 44% and 55% of the original activity being determined after 12-h incubation in the presence of valine, GTP and GTP plus valine, respectively. This observation may be explained by the loss of sensitivity to GTP in the (oxidized GTP) · enzyme derivative, as reported later in the paper.

The inhibition by oxidized GTP can also be reversed by dialysis or gel filtra-

tion; samples which retained 10—20% residual activity regained 50—65% of the original activity after either 48 h dialysis against several changes of 0.8 M KCl/0.02 M phosphate buffer (pH 6.9) or chromatography on a column (800 × 10 mm) of Sephadex G-25 equilibrated with 50 mM imidazole · HCl (pH 7.1), 0.5 M KCl, 25 mM valine and 0.2 mM GTP. The recovery of original activity was only 30% if chromatography was performed in the absence of valine and GTP. Once again, no reactivation was observed with the $NaBH_4$-reduced (oxidized GTP) · enzyme complex.

*Effect of some modifiers of enzymic activity on the inactivation*

The rate as well as the degree of inactivation at low concentrations of oxidized GTP depends strongly on the KCl concentration of the incubation mixture (Fig. 2). After 30 min of incubation, when the reactions are almost complete, inhibitions of 85%, 64%, 37% and 8% are observed in the presence of 0.1, 0.2, 0.4 and 0.6 M KCl respectively. In this and the following experiments the incubation buffer used was 0.04 M succinate/0.4 M Tris · HCl (pH 8.0), which gave the best stability to the enzyme in the presence of low KCl concentrations. However, as is shown by Fig. 2, the controls were not sufficiently stable unless the KCl concentration of the incubation mixture was 0.2 M or higher.

The rate of inactivation by oxidized GTP in the presence of nucleotides which affect the enzyme activity was also investigated. In Fig. 3 the effects of GTP, ATP and ADP are compared. GTP exerts a protective effect which is strongly concentration dependent: AMP deaminase retains 40% and 75% of the original activity when incubation with oxidized GTP is carried out in the presence of 100 μM and 400 μM GTP respectively. 400 μM ATP is as effective as

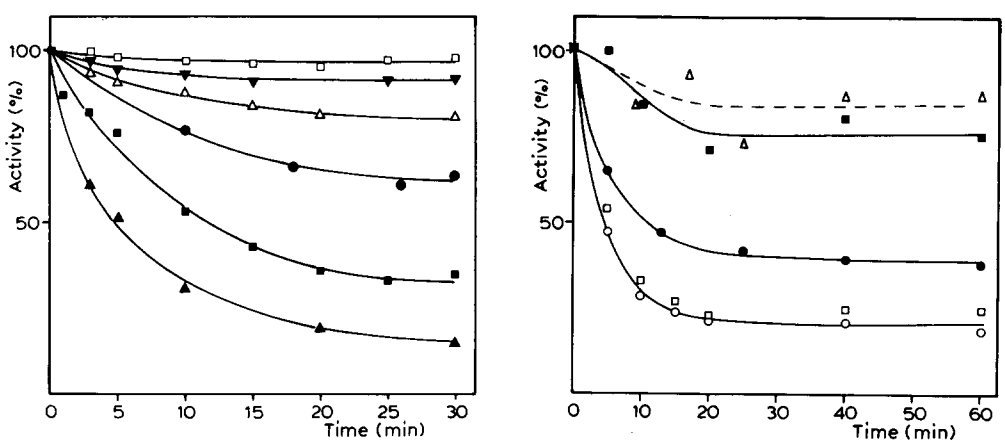



Fig. 2. Effect of KCl concentration on the rate of inactivation of AMP deaminase by oxidized GTP. The enzyme, 0.4 μM, was incubated with 7 μM oxidized GTP in 0.04 M succinate/0.4 M Tris · HCl (pH 8.0), containing 100 mM KCl (▲), or 200 mM KCl (■), or 400 mM KCl (●), or 600 mM KCl (▼). Controls without oxidized GTP with 100 mM KCl (△) or 200 mM KCl (□).

Fig. 3. Effect of some modifiers of AMP deaminase activity on the rate of inactivation by oxidized GTP. The enzyme, 0.6 μM, was incubated with 10 μM oxidized GTP in 0.04 M succinate/0.4 M Tris · HCl (pH 8.0), containing 200 mM KCl (○); 100 μM GTP added (●); 400 μM GTP added (■), 400 μM ATP added (△), 400 μM ADP added (□).

GTP in the enzyme protection, while ADP at the same concentration has no effect.

*Kinetic properties of the (oxidised GTP) · AMP deaminase complex*

The curves of inactivation of AMP deaminase by different concentrations of oxidized GTP at 0.2 M KCl are reported in Fig. 4. In about 60 min the reactions were complete and prolonged incubation did not produce any further appreciable change. Both the rate and the extent of inactivation are dependent on the reagent concentration. Two of the inhibition curves demonstrate inactivation with 6 $\mu$M oxidized GTP, when substrate concentrations both lower (0.1 mM) and higher (2 mM) than $K_m$ (0.5 mM AMP [10]) were used in the assay mixture. The extent of inhibition obtained at the completion of the reaction was 71% and 37% respectively. This observation is related to the nature of the inactivation of AMP deaminase by oxidized GTP, which is essentially due to an allosteric mechanism, as is shown by Figs 5 and 6, which compare the kinetic properties of the native and inactivated enzymes in the presence of 60 mM KCl. For this experiment the (oxidized GTP) · AMP deaminase complexes had been prepared and stabilized by $NaBH_4$ reduction as described in Materials and Methods. Their degree of inactivation was the same as before reduction. This was determined by assaying the native and treated enzymes in the presence of 0.1 mM AMP and 100 mM KCl. Native AMP deaminase, which is completely activated by 60 mM KCl shows an hyperbolic substrate vs. velocity curve, with a Hill coefficient of 1.05. The curves of the 70%- and 85%-inactivated enzymes are sigmoidal with Hill coefficients of 1.3 and 1.5 respectively (Figs. 5 and 6B). The double reciprocal plots (Fig. 6A) show that no noticeable change in $V$ up to 85% inactivation occurs in the treated enzyme, but that the decreased activity results from modification of $K_m$ values (0.5 mM, 1.2 mM and 1.8 mM for the native, 70% and 85% inactivated enzyme, respectively). When assayed at

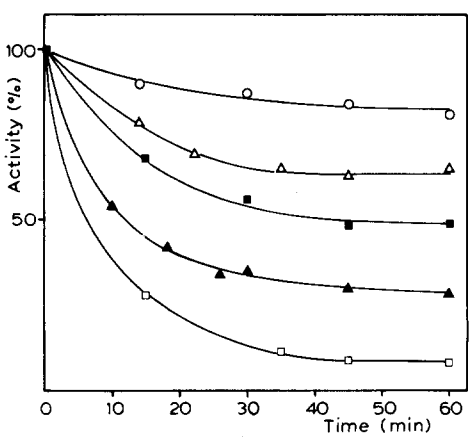



Fig. 4. Effect of oxidized GTP concentration on the rate of inactivation of AMP deaminase. The enzyme, 0.4 $\mu$M, was incubated in 0.04 M succinate/0.4 M Tris · HCl (pH 8.0)/200 mM KCl, with oxidized GTP, 1 $\mu$M (○), 3 $\mu$M (■), 6 $\mu$M (▲) and 12 $\mu$M (□). The enzyme, reacted with 6 $\mu$M oxidized GTP, was also assayed in the presence of 2 mM AMP (△).

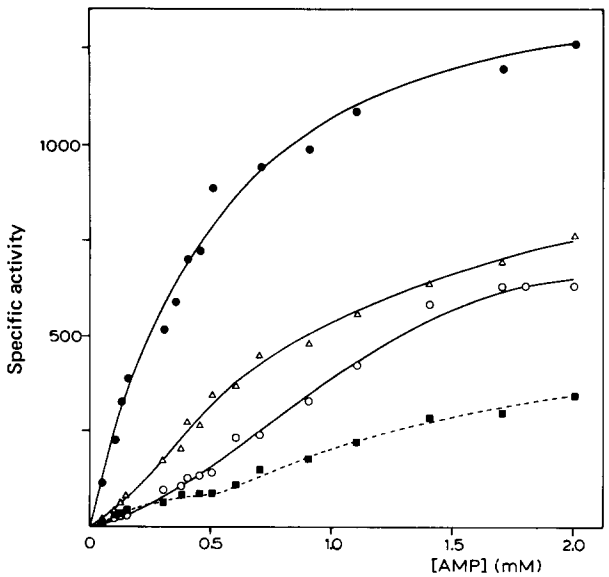



Fig. 5. Substrate vs. velocity curve of native and oxidized GTP-treated AMP deaminases. The reaction mixture contained 60 mM KCl/50 mM imidazole · HCl (pH 6.5) and the reported AMP concentrations. ●, native enzyme; oxidized GTP-treated enzymes at 30% (△), 15% (○) and 8% (■) of the original activity.

60 mM KCl the 92% inactivated enzyme shows at AMP concentrations lower than 0.2 mM a higher activity than that of 85% inactivated enzyme, but no appreciable increase in activity is observed between 0.3 and 0.5 mM AMP (Fig. 5). The corresponding Hill plot did not fit with a straight line, variations in the Hill coefficient being observed from 1.0 in the initial part of the curve to 0.5 in the intermediate plateau and then to values higher than 1.0. This behaviour which may be interpreted as being due to a negative co-operativity effect [14], sug-

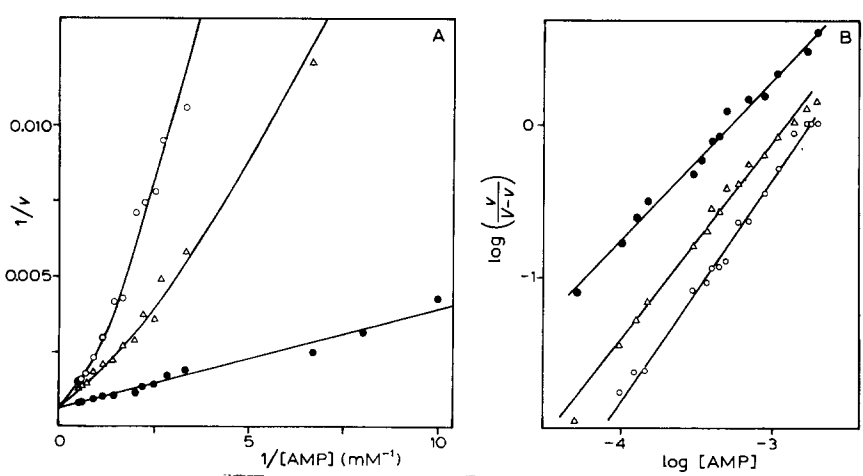



Fig. 6. Lineweaver-Burk and Hill plots of native and oxidized GTP-treated AMP deaminase activities. Double reciprocal plot (A) and Hill plot (B) of kinetic data from Fig. 5. ●, native enzyme; oxidized GTP-treated enzymes at 30% (△) and 15% (○) of the original activity.

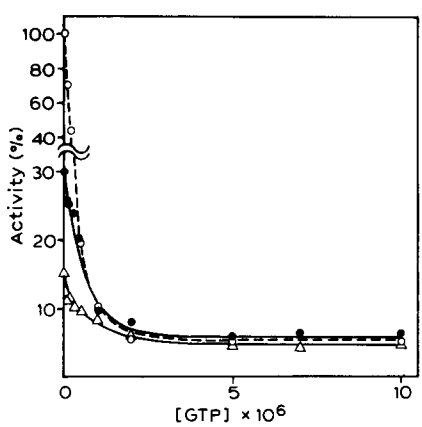
gests that more than one type of site was modified after the extensive treatment of the enzyme with a 30-fold molar excess of oxidized GTP.

GTP, which inhibits the native AMP deaminase to the value of 95% still exerts an inhibitory effect on the reduced (oxidized GTP) · AMP deaminase derivatives with 30% and 15% residual activity (Fig. 7); however, in both cases the value of inhibition is such that the enzyme retains about 5% of the original activity.

*Binding of the periodate oxidation product of GTP by AMP deaminase*

In order to determine the number of mol of oxidized GTP bound per mol of AMP deaminase, the enzyme (0.4—0.5 μM) was treated with different concentrations of the radioactive reagent prepared as described in Materials and Methods. The incubation was carried out in 0.04 M succinate/0.4 M Tris · HCl (pH 8.0) and 200 mM KCl for about 60 min, when no further change in enzyme activity occurred. After $NaBH_4$ reduction and dialysis the radioactivity and the specific activity of the enzyme were determined. The ratio ($\bar{\nu}$) of mol of periodate-oxidized [$^3$H]GTP bound per mol of enzyme at various degrees of inactivation are plotted in Fig. 8, which also shows the residual activities obtained after 60-min incubation with the indicated concentrations of unlabelled oxidized GTP. All the results of Fig. 8 up to 80% loss of activity fit with an exponential decay curve, so that a straight line is obtained when they are plotted semilogarithmically.

The double reciprocal plot of $\bar{\nu}$ as a function of the concentration of free reagent was linear. The slope and intercept and their standard errors were calcu-



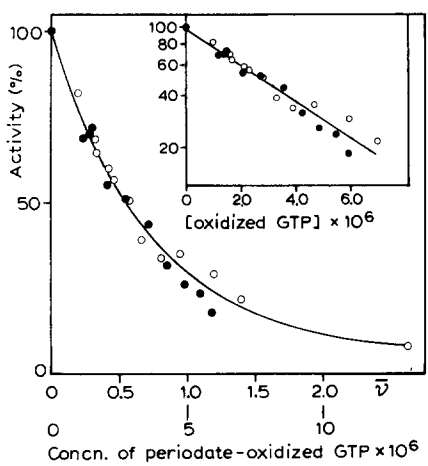




Fig. 7. Effect of GTP on the activity of native and oxidized GTP-treated AMP deaminases. The reaction mixture contained 50 mM imidazole · HCl (pH 6.5), 60 mM KCl and 0.1 mM AMP. ○, Native AMP deaminase; oxidized GTP-treated AMP deaminase at 30% (●) and 15% (△) of the original activity.

Fig. 8. Correlation between AMP deaminase inactivation and binding of oxidized GTP. ○, Residual enzyme activity versus oxidized GTP concentration; ●, Residual enzyme activity versus $\bar{\nu}$ (mol of oxidized [$^3$H]GTP bound per mol of enzyme after $NaBH_4$ reduction). Calculations are based on a specific radioactivity of oxidized [$^3$H]GTP of $13 \cdot 10^7$ cpm/μmol and a molecular weight of AMP deaminase of 290000. Inset, semilog plot of the data.

lated by fitting the data to a straight line by the method of least squares, with a weight $\bar{v}^4$ for each point to allow for the effect of taking reciprocals [15]. The number of binding sites so determined corresponded to 1.94 ± 0.11 mol of oxidized GTP/290000 g of protein, with an apparent dissociation constant of 6.79 ± 0.74 $\mu$M.

## Discussion

The inhibitory effect of GTP on skeletal muscle AMP deaminase has been extensively studied [2,4,5,16]. This nucleotide behaves as an allosteric effector causing a strong inhibition of the enzyme at low concentrations of substrate without affecting the $V$ value, so that in the presence of GTP a positive homotropic cooperativity for the substrate is observed. The results presented in this paper show that the GTP binding sites of rat-muscle AMP deaminase can be specifically and reversibly modified by periodate-oxidized GTP. The inhibition of enzymic activity observed after oxidized GTP treatment can be reversed by dialysis against solutions free of the inhibitor or by addition of amino acid solutions, indicating that an equilibrium exists between the (oxidized GTP) · protein complex and the free inhibitor and protein; this equilibrium can be shifted towards the fully active free AMP deaminase by removing the unbound oxidized GTP either by dialysis or reacting it with amino groups. A faster rate of reactivation is obtained if the process is carried out in the presence of GTP. The observation that, after $NaBH_4$ treatment, the bound inhibitor cannot be removed from the protein, strongly suggests that in the binding of oxidized GTP to AMP deaminase a Schiff base is formed between the aldehyde groups of the inhibitor and amino groups of the enzyme.

The binding data obtained from the investigation carried out with the radioactive reagent fit with an hyperbolic saturation curve which indicates binding of 1.9 mol of oxidized GTP per 290000 g of protein, with an apparent dissociation constant of $6.8 \cdot 10^{-6}$ M. This result is in good agreement with the previous observation of Tomozawa and Wolfenden [5], who found 2 binding sites for GTP with an apparent dissociation constant of $2 \cdot 10^{-6}$ M by equilibrium binding studies with rabbit back muscle AMP deaminase.

The suggestion that periodate-oxidized GTP specifically modifies the binding sites for GTP is supported by the kinetic behaviour of the reduced (oxidized GTP) · enzyme derivative which shows positive homotropic cooperativity for the substrate even in the presence of 60 mM KCl, when the native enzyme substrate vs. velocity curve is hyperbolic. Furthermore, the modified enzyme shows a loss of sensitivity to GTP corresponding to its degree of inactivation.

The rate of AMP deaminase inactivation by oxidized GTP is affected by some enzyme modifiers. A strong protection is shown by KCl, which at high concentrations is known to influence the regulatory properties of skeletal muscle AMP deaminase by decreasing the effect of activators and inhibitors [2,4]. It has been reported that the rabbit muscle enzyme shows a KCl-dependent association that has been postulated to take part in the regulatory mechanism of the enzyme [17]. On this basis it is likely that KCl may prevent binding of oxidized GTP as well as of effectors inducing a structural change in the AMP deaminase molecule. Interestingly, KCl does not protect the rat muscle

enzyme from the inactivation by dinitrophenyl fluoride [18] which is due to modification of tyrosine residues in the enzyme active centre [19].

A protective effect against inactivation by oxidized GTP is exerted also by nucleoside triphosphates, while ADP at the same concentration is without effect. The observation that ATP, as well as GTP, protects the enzyme against inactivation by oxidized GTP can be explained either by the existence of common binding sites for these two nucleotides or by a conformational change elicited by ATP which no longer permits binding of oxidized GTP. The first interpretation would be consitstent both with the mutually inhibitory binding of ATP and GTP to rabbit muscle AMP deaminase [5] and with the conclusion drawn from the results of a study on the kinetic properties of rat muscle AMP deaminase treated with pyridoxal-5′-*P* [3]. It was inferred that the modification of 6—7 lysines with pyridoxal-5′-*P* "freezes" the enzyme in the conformational state of the native enzyme in the presence of nucleoside triphosphate (ATP as well as GTP). The kinetic behaviour of this derivative was quite similar to that presented in this paper for the periodate-oxidized GTP-modified enzyme. However, the possibility that the protection exerted by ATP against the binding of oxidized GTP may be, at least partly, due to an allosteric mechanism cannot be excluded. A more detailed kinetic study is now in progress to distinguish between these possible effects of ATP on oxidized GTP-induced inactivation.

It has been mentioned above that after extensive treatment with a 30-fold molar excess of oxidized GTP rat muscle AMP deaminase retains less than 10% of the original activity and shows negative homotropic cooperativity. This degree of inactivation is achieved after a deviation from linearity in the semilog plot of the inactivation data is observed, indicating that it is probably due to the modification of sites different from those specific for GTP. The possibility that the modification of sites specific for ATP occurs at this level of inhibition is supported by the observation that in the presence of ATP negative homotropic cooperativity is observed in rat muscle AMP deaminase [20]. Preliminary results indicate that the same kinetic behaviour is shown by the enzyme treated with periodate-oxidized ATP and AMP.

It was not the aim of this paper to decide whether or not GTP and ATP bind at common sites on the AMP deaminase molecule; however, the finding that treatment with periodate-oxidized nucleotides can specifically modify the sites of either of these inhibitors forms the basis for further studies to clarify the regulatory properties of the enzyme.

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