Inactivation of rat muscle 5'-adenylate aminohydrolase by tyrosine nitration with tetranitromethane

Maria RANIERI-RAGGI, Carlo BERGAMINI,* Umberto MONTALI and Antonio RAGGI
Istituto di Chimica Biologica dell'Università di Pisa, 56100 Pisa, Italy

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Reaction of rat muscle AMP deaminase with low molar excess of tetranitromethane results in a rapid loss of free thiol groups and a concomitant decrease in enzyme activity at high, but not at low, AMP concentration. This modification appears to be limited to the same non-essential thiol groups reactive towards specific reagents in non-denaturing conditions. On incubation with higher molar excess of tetranitromethane, a loss of enzyme activity is observed, which correlates with nitration of tyrosine residues. By amino acid analysis, approximately three tyrosine residues per subunit are estimated to be nitrated in the completely inactivated enzyme. The kinetic properties of the partially inactivated AMP deaminase reveal a negative co-operativity behaviour at approximately half saturation. This suggests that modification of tyrosine residues is also responsible for alteration of the binding properties of the hypothesized activating site of AMP deaminase.

The use of reagents for specific modification of amino acid residues in proteins has been a fruitful means of determining the chemical structure of ligand-binding sites. This approach is particularly interesting with regulatory enzymes, where both the allosteric interactions and the catalytic process could selectively be affected by chemical modification. Previous investigations in our laboratory have demonstrated this possibility to be feasible for rat skeletal-muscle AMP deaminase (AMP aminohydrolase, EC 3.5.4.6). In this enzyme the sensitivity to allosteric inhibition by nucleoside triphosphates can be influenced by modifying either cysteine or lysine residues. Partial desensitization towards ATP and GTP was observed in AMP deaminase after modification of the thiol groups accessible to thiol reagents in the native protein (Raggi *et al.*, 1972). The specific modification of nucleoside triphosphate-binding sites in AMP deaminase was shown to be the results of modification of 6–7 lysine residues by pyridoxal phosphate and NaBH_4 , occurring without an appreciable decrease in V (Ronca-Testoni & Ronca, 1974). Two more recent papers have confirmed the involvement of lysine residues in the regulatory sites of the enzyme, by means of affinity labelling with periodate-oxidized ATP and GTP

Abbreviations used: Dnp-, 2,4-dinitrophenyl-; Dnp-F, 1-fluoro-2,4-dinitrobenzene; Nbs₂, 5,5'-dithiobis-(2-nitrobenzoic acid); Nbs, 2-nitro-5-thiobenzoic acid.

* Present address: Istituto di Patologia Generale dell'Università di Pisa, 56100 Pisa, Italy

(Ranieri-Raggi & Raggi, 1976; Raggi & Ranieri-Raggi, 1979). On the other hand, dinitrophenylation of about six tyrosine and two lysine residues completely inactivated *N*-ethylmaleimide-treated AMP deaminase (Raggi *et al.*, 1975). During thiolysis of Dnp-enzyme with 2-mercaptoethanol, much of the enzyme activity was restored, suggesting that modification of tyrosine residues was responsible for the inactivation.

In the present study we have further investigated the participation of tyrosine residues at the active site of rat muscle AMP deaminase by subjecting the enzyme to modification with tetranitromethane, a reagent considered to affect tyrosine residues with greater specificity than Dnp-F. It will be shown that AMP deaminase is inactivated by tetranitromethane with a time course which depends on the state of thiol groups in the protein. In any case, almost complete inactivation of AMP deaminase is obtained when 2–3 tyrosine residues per subunit are nitrated. These results confirm the suggestion of an obligatory participation of tyrosine residues in the enzyme active centre.

Materials and methods

Enzyme

Rat skeletal-muscle AMP deaminase was purified as previously described (Ranieri-Raggi & Raggi, 1976). The preparation gave a single band of mol.wt. $70\,000 \pm 2\,000$ when $50\,\mu\text{g}$ was electrophoresed on

10% polyacrylamide gel in 0.1% sodium dodecyl sulphate in 0.1 M-Tris/0.1 M-Bicine [*N,N*-bis-(2-hydroxyethyl)glycine] (Sigma Chemical Co., St. Louis, MO, U.S.A.) (pH 8.3), by the method of Weber & Osborn (1969). The specific activity of the enzyme was 1200 μmol of AMP deaminated/min per mg of protein at 20°C in 50 mM-imidazole/HCl (pH 6.5)/60 mM-KCl/2 mM-AMP (Sigma). The molecular weight of the enzyme was taken as 290 000 (Ronca-Testoni *et al.*, 1970; Ogasawara *et al.*, 1977). Protein concentration was calculated from the A_{280} by using an $A_{1\text{cm}}^{1\%}$ value of 9.8 (Ronca-Testoni *et al.*, 1970). AMP deaminase activity was routinely determined spectrophotometrically as described by Ronca *et al.* (1968), in 50 mM-imidazole/HCl (pH 6.5)/100 mM-KCl.

Inactivation studies

Incubation of the enzyme with different concentrations of tetranitromethane (Fluka A. G., Buchs, Switzerland) was carried out at 20°C in 0.01 M-succinate/0.1 M-Tris/HCl (pH 8.0)/0.8 M-KCl (buffer A). The reaction was started by adding to the incubation mixture 2 μl of a 95% (v/v)-ethanol solution of freshly thawed tetranitromethane (stored at -20°C). At intervals, samples of the reaction mixture were removed and immediately tested for AMP deaminase activity by using a 1000-fold dilution in 50 mM-imidazole/HCl (pH 6.5)/100 mM-KCl/0.1 mM-AMP (or 2 mM-AMP when specified). Control samples were incubated in buffer in the absence of tetranitromethane. The reaction of AMP deaminase with tetranitromethane was terminated by lowering the temperature to 0°C. For further studies this mixture was extensively dialysed against 5 mM-Tris/HCl (pH 7.0) containing 1 M-KCl (buffer B) to remove from the enzyme the other products of the reaction. For analysis of the time course of nitration, the A_{428} of the incubation mixture was measured at intervals and the nitrotyrosine content was calculated by using an absorption coefficient of 4100 litre·mol⁻¹·cm⁻¹ (Sokolovsky *et al.*, 1966). The number of nitrotyrosine residues was also estimated from absorbance measurements on the dialysed enzyme at the isosbestic point, i.e. at 381 nm, by using an absorption coefficient of 2200 litre·mol⁻¹·cm⁻¹ (Sokolovsky *et al.*, 1966). A Cary model 14 automatic recording spectrophotometer was employed to obtain continuous absorption spectra. The other spectrophotometric determinations were carried out in a Zeiss PMQ II spectrophotometer equipped with a Zeiss TE converter and with a Varian 2000 recorder. All the reagents used were analytical grade.

Analytical measurements

Titration of AMP deaminase thiol groups with Nbs₂ (Sigma) were carried out at room temperature

as described by Ellman (1959). The reaction mixture contained 0.3 mM-Nbs₂, 0.1 M-Tris/HCl (pH 8.0), 0.8 M-KCl and 1.6–1.9 μM -AMP deaminase. Amino acid analyses were performed by the procedure of Moore & Stein (1963), with a Beckman Unicrom amino acid analyser. Both native and nitrated AMP deaminases were dialysed for 24 h against several changes of 50 mM-NH₄HCO₃, pH 8.2, freeze-dried and hydrolysed with 6 M-HCl in evacuated sealed tubes for 24 h at 110°C. Quantitative determinations of nitrotyrosine were obtained from amino acid analysis as described by Sokolovsky *et al.* (1966).

Results

Reaction of AMP deaminase with tetranitromethane

Incubation of AMP deaminase with tetranitromethane at pH 8.0 (Fig. 1) results in a progressive loss of enzyme activity assayed at low substrate concentration (0.1 mM-AMP). Both the reaction rate and the shape of the inactivation curve depend on the reagent concentration. Although tetranitromethane concentrations higher than 1 mM completely inactivated the enzyme at a first-order rate, at lower reagent concentrations the loss of activity was biphasic, the first 2–3 min of incubation being followed by a higher inactivation rate. An initial lag phase was constantly observed in several experiments when tetranitromethane was used at a

Fig. 1. Inactivation of rat muscle AMP deaminase by tetranitromethane

The enzyme (2 μM) was incubated in buffer A with tetranitromethane, 0.3 mM (circles) or 0.8 mM (triangles); control samples without tetranitromethane (squares). Full and empty symbols refer to native and Nbs₂-treated enzymes respectively.

concentration lower than 0.5 mM (Fig. 1). This phenomenon is not observed when the reaction is performed with an AMP deaminase sample previously incubated with Nbs_2 to modify the 12 cysteine residues that are not essential for enzymic activity (Raggi *et al.*, 1972). Under these particular conditions, AMP deaminase is immediately inactivated by tetranitromethane, even at a reagent concentration as low as 0.3 mM. This results in a faster rate of disappearance of enzyme activity as compared with that of native enzyme, residual activities after 24 min of incubation with 0.3 mM- and 0.8 mM-tetranitromethane being 63% and 20% for native enzyme and 48% and 4% for Nbs_2 -treated AMP deaminase respectively.

Modification of tyrosine residues is responsible for inactivation

In several proteins, tetranitromethane is known to react specifically with tyrosine residues, which are modified to residues of highly chromophoric 3-nitrotyrosine; to test the formation of this product we have analysed the spectral properties of tetranitromethane-treated AMP deaminase at different pH values. In this experiment, $2\text{ }\mu\text{M}$ - Nbs -enzyme was incubated with $400\text{ }\mu\text{M}$ -tetranitromethane for 30 min, with a 90% loss of catalytic activity. After extensive dialysis against buffer B containing 1 mM-2-mercaptoethanol, continuous absorption spectra were recorded at this pH, and at pH 8.0 and 10.0 adjusted by addition of 1 M-Tris base. Recordings presented in Fig. 2 show the intersection of the spectra with an isosbestic point at 381 nm and the appearance at alkaline pH of a new absorbance maximum in the visible region, with peak value at

428 nm. Both these properties are in agreement with the characteristics previously described for the reaction product of tetranitromethane with tyrosine residues (Sokolovsky *et al.*, 1966).

To correlate the extent of protein modification with the loss of catalytic activity, we compared the amino acid content of native AMP deaminase with that of two samples of tetranitromethane-treated enzyme at different degrees of inactivation. Tyrosine was the only amino acid decreased, beyond the experimental error, as a result of nitration. In native AMP deaminase 122 ± 1.8 tyrosine residues were determined, in agreement with the value of 119 reported by Coffee & Kofke (1975), recalculated to moles per 290 000 g of enzyme, and the modified enzyme at 44% and 21% of the original activity contained 114 and 112 tyrosine residues respectively. The loss of about 8 tyrosine residues observed in the less-inactivated AMP deaminase is accounted for by the number of nitrotyrosine residues determined in the same sample either by amino acid analysis or spectrophotometrically at 381 nm after dialysis. At the higher degree of inactivation, a somewhat higher nitrotyrosine content was obtained by the absorbance method than by amino acid analysis (Table 1). The number of nitrotyrosine residues produced by reaction of Nbs_2 -treated AMP deaminase with tetranitromethane did not differ significantly from that obtained with the native enzyme when the determination was based on the A_{381} of the dialysed enzyme. On the contrary, a remarkable difference was observed when the number of nitrotyrosine residues was estimated directly from the A_{428} of the incubation mixture. This is shown in Fig. 3, where the experimental results were plotted on a semilogarithmic scale against the extent of inactivation. Although a linear correlation is observed between inactivation of the Nbs_2 -treated

Fig. 2. Influence of pH on absorption spectrum of nitrated AMP deaminase
AMP deaminase was nitrated with tetranitromethane as described in the text.

Table 1. Quantification of residues of nitrotyrosine in tetranitromethane-treated AMP deaminase

Two samples of AMP deaminase (6 nmol) were treated for different times with a 400-fold molar excess of tetranitromethane in 2.5 ml of buffer A. The reported residual activity values were determined at the end of dialysis against buffer B. For amino acid analysis, the two samples were further processed as described in the Materials and methods section.

AMP deaminase residual activity (%)	Nitrotyrosine (mol/mol of AMP deaminase)	
	By A_{381}	By amino acid analysis
44	8.4	6.7
21	18.0	10.5

Fig. 3. Correlation between residual AMP deaminase activity and apparent number of tyrosine residues nitrated

Nitrotyrosine (NO_2Tyr) content was determined in different experiments from the A_{428} of the incubation mixture during the reaction of native ($\circ$) and Nbs_2 -treated ($\blacktriangle$) AMP deaminases with 200–500-fold molar excess of reagent. AMP deaminase activity was assayed as described in the Materials and methods section.

enzyme and the number of nitrotyrosine residues calculated, for native enzyme an initial increase in A_{428} is obtained without a concomitant loss of activity. This observation can be explained by the reaction of tetranitromethane with amino acids other than tyrosine that are not essential for enzyme activity. The fact that in proteins tetranitromethane may be reactive towards cysteine as well as tyrosine residues (Riordan & Vallee, 1972) and our previous identification of 12 thiol groups not essential for activity of AMP deaminase (Raggi *et al.*, 1972) prompted us to evaluate the modification of cysteine residues during the inactivation process. Titration with Nbs_2 in non-denaturing conditions at various stages of the tetranitromethane reaction gave values of less than 1 thiol group per molecule already in the 10%-inactivated enzyme (results not shown). This indicated a rapid modification by tetranitromethane of the non-essential thiol groups of AMP deaminase. We quantified this reaction by using the absorption coefficient of $600\text{ litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ that we determined for the reaction product of tetranitromethane and cysteine at 428 nm and pH 8.0. Assuming that the mechanism of the reaction of tetranitromethane with thiol groups in AMP deaminase is that described for cysteine (Sokolovsky *et al.*, 1969), the initial increase in A_{428} without loss of activity can be accounted for by the modification of approx. 16 thiol groups per enzyme molecule.

Modification of 12 thiol groups of AMP deaminase with Nbs_2 or *N*-ethylmaleimide inhibits the enzyme activity at high, but not at low, substrate concentration (Raggi *et al.*, 1972, 1975; the present paper, see Fig. 6). Therefore it was decided to extend the investigation of the effects of the incubation with a low tetranitromethane concentration (0.2 mM) on AMP deaminase activity at saturating substrate concentration. Under these assay conditions, no lag phase is observed in the inactivation of native as well as of Nbs_2 -treated AMP deaminase (Fig. 4). The two enzymes show about the same specific activity at the end of the treatment although the initial activity of Nbs -enzyme was only 60% of that of native AMP deaminase. This clearly indicates that modification of thiol groups by either tetranitromethane or Nbs_2 has the same effect on AMP deaminase kinetics. Further examination of the data of Fig. 4 reveals that for the Nbs -enzyme the loss of activity at 0.1 mM-AMP is significantly higher than that at 2 mM-AMP, residual activities after 22 min of incubation being 25% and 45% of the respective initial values. This suggests that nitration of tyrosine residues is also responsible for alteration of the kinetic parameters of the enzyme.

Effect of nitration on the kinetic properties of AMP deaminase

The above observations indicate that during the

Fig. 4. Effect of tetranitromethane on AMP deaminase activity at high and low AMP concentrations

Native (triangles) and Nbs_2 -treated (circles) AMP deaminases ($1.7\ \mu\text{M}$) were incubated with $200\ \mu\text{M}$ -tetranitromethane in buffer A. At intervals samples were assayed for residual activity at 0.1 mM-AMP (full symbols) or 2 mM-AMP (empty symbols). At both substrate concentrations, the 100% value is assigned to the specific activity of native enzyme at zero time of incubation.

treatment of AMP deaminase with tetranitromethane two distinct phenomena take place: the overall inactivation of the enzyme, brought about by extensive nitration, and the alteration of the kinetic properties during mild modification, as demonstrated by comparison of the pattern of inactivation of the enzyme assayed at low and high substrate concentrations. The presence of both these phenomena makes difficult to ascribe the loss of activity to modification of either essential amino acid residues or non-essential ones, i.e. not participating at the active site but influencing the kinetic behaviour. To solve this point we investigated the substrate saturation of the enzyme at different degrees of inactivation, measured at 0.1 mM-AMP. Independently of the extent of treatment, the incubation of AMP deaminase with tetranitromethane induces a modification of the enzyme kinetics, such that the double-reciprocal plot of the data (Fig. 5) shows a downwards inflexion at intermediate (around 0.4 mM-AMP) substrate concentration. Consequently, two different K_m values can be determined according to the activity values obtained at either low or high AMP concentration. In the first case, the tetranitromethane-treated enzymes show a K_m value (0.1–0.2 mM-AMP) lower than that of native AMP deaminase (0.5 mM-AMP), whereas at higher substrate concentration the modified enzyme appears to bind substrate with a

lower affinity, K_m being approx. 1 mM AMP. Downwards curvature in Lineweaver–Burk plots could be explained by the presence of high- and low- K_m enzyme species. However, since the point of inflexion does not move as the extent of modification increases, a more likely interpretation for the higher degree of inactivation observed at intermediate substrate concentration (0.4–0.5 mM-AMP) is that treatment with tetranitromethane induces the transition of AMP deaminase to a form exhibiting negative co-operativity.

The data reported in Fig. 4 on the progression of the inactivation by tetranitromethane of the Nbs₂-treated enzyme show a faster inhibition of activity at 0.1 mM- than at 2 mM-AMP, different from what is observed during the treatment of the native enzyme. Therefore it cannot be excluded that thiol modification by tetranitromethane might be at the basis of the kinetic properties of the partially inactivated AMP deaminase reported above. To test this hypothesis, we investigated the effects of tetranitromethane in an AMP deaminase sample whose reactive thiol groups were protected by modification with Nbs₂. The enzyme treated with Nbs₂ to block the reactive thiol groups completely was subjected to reaction with tetranitromethane until 90% inactivation at 0.1 mM-AMP was reached. The kinetic properties of this enzyme did not substantially differ from those of the enzyme inactivated by tetranitromethane without previous protection of thiol groups. In fact, for the nitrated Nbs-enzyme also the shape of the substrate-saturation curve is typical of negative homotropic co-operativity at intermediate substrate concentration (Fig. 6).

To a sample of this tetranitromethane-inactivated Nbs-enzyme, 2-mercaptoethanol was added to a concentration of 1 mM. This mixture was dialysed for 24 h against several changes of 1 mM-2-mercaptoethanol in buffer B to recover thiol groups in the native state. 2-Mercaptoethanol was then removed by extensive dialysis against buffer B and the spectrophotometric determination of free thiol groups of AMP deaminase was performed with Nbs₂ as described in the Materials and methods section. The titration gave the value of 7.6 thiol groups per molecule of AMP deaminase. This number is somewhat lower than that of approx. 12 modified by Nbs₂ in the native enzyme. This difference may be explained by a lower reactivity of either Nbs groups towards 2-mercaptoethanol or thiol groups towards Nbs₂ as a consequence of the nitration of AMP deaminase. The effect of displacing Nbs groups on the kinetic properties of tetranitromethane-inactivated Nbs-AMP deaminase is shown in Fig. 6; thiolysis with 2-mercaptoethanol apparently removes the inhibitory effect at high substrate concentration without any other major modification in the shape of the substrate-saturation curve.

Fig. 5. Lineweaver–Burk plots of native and tetranitromethane-treated AMP deaminase activities

The reaction mixture contained 50 mM-imidazole/HCl (pH 6.5) and 100 mM-KCl. Tetranitromethane-treated enzymes at 15 (▲), 50 (○) or 90% (■) of the original activity; △, native enzyme.

Fig. 6. Influence of the state of reactive thiol groups on substrate-saturation curve of tetranitromethane-treated AMP deaminase

Nbs₂-treated AMP deaminase was inactivated by tetranitromethane to 10% of the original activity and assayed before (○) and after (●) treatment with 2-mercaptoethanol as described in the text. △, native AMP deaminase; ▲, Nbs₂-treated AMP deaminase. Assays were performed at 20°C in 100 mM-KCl/50 mM-imidazole/HCl (pH 6.5). One unit of specific activity is the amount of enzyme that catalyses the deamination of 1 μmol of AMP/min per mg of protein.

Discussion

Nitration with tetranitromethane has emerged as the most useful method with which to study the role of tyrosine residues in the biological activity of proteins. This opportunity derives mainly from the property of tetranitromethane to be a quite specific reagent, only tyrosine and cysteine residues being affected under mild conditions; other amino acids are seldom modified during more drastic treatment, i.e. methionine, tryptophan and histidine (Sokolovsky *et al.*, 1970; Riordan & Vallee, 1972).

Our present paper describes the results that we obtained by incubating rat muscle AMP deaminase with tetranitromethane. During this treatment, both the catalytic and the modulatory properties of the enzyme are affected, as demonstrated by the changes in the kinetic parameters: together with the decrease in V , an indication of an inactivation process, a modification of K_m is observed, suggesting an altered co-operative pattern of the residual enzyme activity. Since the changes in the kinetic properties of AMP deaminase during the incubation show two distinguishable phases, their analysis deserves consideration in establishing the nature of the groups involved in the modification of the enzyme. The early events taking place are manifested functionally as a decreased enzyme activity at saturating AMP concentrations, whereas that at substrate concentrations lower than K_m remains unchanged. This phenomenon resembles the kinetic

effects of modification of 12 non-essential thiol groups of AMP deaminase by specific reagents (Raggi *et al.*, 1972). The involvement of thiols also in this case is suggested by the fact that the absorbance increase of the incubation mixture during the first phase of treatment with tetranitromethane can be ascribed to the oxidation of approx. 16 thiol groups and that thiol groups are no longer available to react with Nbs₂ in non-denaturing conditions. That the cysteine residues modified are not involved in the true inactivation process is confirmed by the observation that their modification with Nbs₂ does not protect the enzyme from inactivation by tetranitromethane; on the contrary, a faster disappearance of activity at low AMP concentration is observed for Nbs-enzyme than for native enzyme.

The second phase of the reaction of tetranitromethane with native AMP deaminase, which leads to the complete loss of enzyme activity at substrate concentrations both higher and lower than K_m , displays a linear relationship between extent of inactivation and nitration of tyrosine residues. Amino acid analysis and spectrophotometric determination of nitrotyrosines demonstrate that in our experimental conditions approx. 5 and 10 tyrosine residues out of a total of 122 are nitrated in the 50% and 80%-inactivated enzymes respectively. Assuming four identical subunits for rat muscle AMP deaminase (Coffee & Kofke, 1975; Ogasawara *et al.*, 1977; the present paper, see the Materials and methods section), these data indicate that approx. 3 tyrosine residues per subunit are nitrated in the 100%-inactivated enzyme. By coupling this observation with the results of Dnp-F treatment, when 1.5 *O*-Dnp-tyrosine residues per subunit were formed at 100% inactivation (Raggi *et al.*, 1975), we may confirm the hypothesis of an essential role of tyrosine residues, probably one per subunit, in the kinetic mechanism of AMP deaminase.

Incubation of AMP deaminase with tetranitromethane brings about, together with the inactivation process, an alteration of the catalytic properties of the enzyme, which no longer follows hyperbolic kinetics, but shows negative co-operativity at AMP concentrations giving approximately half-saturation of the enzyme. Since negative co-operativity is elicited by tetranitromethane even in the Nbs₂-treated enzyme, we can exclude a role of the reactive thiol groups in this phenomenon. Negative co-operativity behaviour was shown by AMP deaminase after the early phase of arylation with Dnp-F, when no decrease of V was observed, only few of the reactive thiol groups being modified (Raggi *et al.*, 1975). Although amino as well as phenolic hydroxy groups of AMP deaminase were dinitrophenylated, the induction by this treatment of the same kinetic effect observed with nitration by

tetranitromethane suggests that negative co-operativity is essentially due to modification of tyrosine residues. In conclusion, the above considerations give evidence for the existence in AMP deaminase of a second class of tyrosine residues susceptible to reaction with both Dnp-F and tetranitromethane in addition to those essential for catalytic activity. Since tetranitromethane, possibly owing to its high reactivity, gives rise to an extent of modification higher than that obtained with Dnp-F, we cannot establish with certainty the number of tyrosine residues belonging to the two classes. However, we may reasonably suggest that AMP deaminase subunit contains one essential tyrosine residue and one or two that are not essential, but are important for the co-operativity phenomena.

It has been previously reported that the effects of salts on the kinetic properties of rat muscle AMP deaminase may be explained by assuming that anionic compounds (including AMP) must bind to the enzyme for its complete activation (Ronca *et al.*, 1972). This hypothesis has also been advanced for the rabbit enzyme (Ashby & Frieden, 1978). At salt concentrations higher than those required for the complete enzyme activation, anions inhibit AMP deaminase to a limited extent, apparently competing with substrate at the activating but not at the catalytic site (Ronca *et al.*, 1972). We have more recently shown that the inhibitory effect of high concentrations of anions, including ATP, is such that AMP deaminase displays negative homotropic co-operativity kinetics (Raggi & Ranieri-Raggi, 1979; Ranieri-Raggi *et al.*, 1980). Since we did not detect any change in the aggregation pattern of AMP deaminase as an effect of chemical modification of tyrosine residues or high salt concentration (A. Raggi, unpublished work), we can assume as a working hypothesis that negative co-operativity in AMP deaminase derives from a conformational alteration influencing the binding properties of the activating site.

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