Modulation of the Kinetic Properties of Phosphofructokinase by Ammonium Ions*

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SUMMARY

Ammonium ions were shown to be much more efficient than potassium ions in activating rabbit skeletal muscle phosphofructokinase (EC 2.7.1.11). Under experimental conditions simulating physiological ones (pH 7.2, and inhibitory concentrations of ATP), the apparent dissociation constant of the phosphofructokinase-NH₄⁺ complex was 0.33 mM. This molarity was shown to lie within the physiological concentration of ammonia in several tissues exhibiting pronounced glycolytic activity. The activation of the enzyme by NH₄⁺ was very specific and rapid. It was observed also when ITP was the phosphate donor, indicating that the activation was not mere deinhibition of ATP.

The form of phosphofructokinase predominant in the presence of NH₄⁺ exhibited in a qualitative fashion the same allosteric characteristics of the enzyme form prevailing in its absence, i.e., sigmoidity with respect to fructose 6-phosphate, and sensitivity to inhibition by citrate and higher concentrations of ATP. However, NH₄⁺ (2 mM) decreased the Kₘ for ATP (from 0.031 mM to 0.013 mM) and for fructose 6-phosphate (from 0.34 mM to 0.04 mM), and increased the Kₘ for citrate (from 0.025 mM to 0.055 mM) and for ATP (from 0.31 mM to 0.48 mM).

It is proposed that the activation of phosphofructokinase by NH₄⁺ could be a regulatory mechanism which provides the metabolites necessary for ammonia fixation and the maintenance of its concentration at tolerable levels. Moreover, the possibility that the increase in tissue concentrations of NH₄⁺ under anoxia might be a contributing factor to the “Pasteur effect” is discussed.

Muntz was the first to show that ammonium ions activated phosphofructokinase (EC 2.7.1.11) in yeast extracts (1), rat brain homogenates (2), and the partially purified enzyme from bovine brain (3). Later, NH₄⁺ was shown to stimulate purified phosphofructokinase from sheep brain (4), rabbit muscle (5, 6), and yeast (7, 8). However, a thorough study of the modulation of catalytic and allosteric properties of rabbit skeletal muscle phosphofructokinase by NH₄⁺ has not been reported. Also, the opinion was expressed (6, 9) that the activation of this enzyme by NH₄⁺ was not physiological in view of the fact that “toxic” concentrations were required. We should like to report here that when the ammonium sulfate present in commercial preparations of auxiliary enzymes used in the assay method is thoroughly removed, muscle phosphofructokinase is stimulated more than 4-fold by readdition of physiological concentrations of NH₄⁺. Also, a physiological role for this effect is proposed.

EXPERIMENTAL PROCEDURE

Materials—Rabbit muscle phosphofructokinase was prepared according to the method of Ling, Marcus, and Lardy (10). Since ATP changes the kinetic characteristics of phosphofructokinase, the crystallization step in presence of this nucleotide was avoided. The enzyme was stored as a concentrated solution (about 20 mg per ml) in 0.1 M potassium phosphate buffer (containing 1 mM EDTA), pH 8.0. It was stable at 4° for several months. The homogeneity of each preparation was established by ultracentrifugation in the presence of 6 M guanidinium chloride. The auxiliary enzymes, namely aldolase, triose phosphate isomerase, and α-glycerophosphate dehydrogenase, were purchased from Boehringer Mannheim Corporation. In order to eliminate thoroughly all traces of NH₄⁺, a mixture of these enzymes was dialyzed for 36 hours against three changes of Tris-chloride (0.05 M) buffer containing EDTA (0.05 mM), pH 8.0. The sodium salts of the substrates fructose 6-phosphate, ATP, and ITP were obtained from Sigma. Highest grade ammonium chloride was used as the NH₄⁺ salt; the chloride ions had no effect on the activity of phosphofructokinase.

Phosphofructokinase Activity—This was assayed by the coupled procedure described by Ling et al. (11). The stock enzyme preparation was diluted with a solution containing Tris (0.05 M), EDTA (1 mM), dithiothreitol (5 mM), bovine serum albumin (0.1%) and adjusted to pH 8.0 with phosphoric acid. An enzyme unit was defined as the amount of phosphofructokinase which catalyzed the conversion of 1 μmole of fructose 6-phosphate to fructose 1,6-diphosphate per min at pH 7.2 and 28°. The phosphofructokinase preparations used had specific activities (approximately 130 to 150 units per mg of protein) comparable to those reported in the literature (10) when assayed under optimal conditions. The lower specific activities reported...
Fig. 1. Activity of phosphofructokinase as a function of ATP and NH$_4^+$ concentrations. The reaction mixture contained 40 mM sodium glycylglycine buffer (pH 7.2), 0.1 mM fructose 6-phosphate, 2 mM MgCl$_2$, and 3 mM dithiothreitol. The $K_m$ for ATP as substrate was determined from Lineweaver-Burk plots using ATP concentrations below 0.2 mM. The $K_i$ for ATP as inhibitor was taken as the ATP concentration which brought about an initial velocity equal to half-$V_{max}$ using the descending phase of the curves (i.e. nucleotide concentrations above 0.2 mM). Other conditions for the assay were as described under “Experimental Procedure.”

Here are due to the use of suboptimal conditions. In particular, the suboptimal pH of 7.2 was used to simulate the physiological condition and to demonstrate clearly the allosteric properties of the enzyme which are obscured at higher pH values.

The enzymatic activity was assayed by a Gilford recording spectrophotometer. The full width of the chart was set for 0.5 absorbance unit in order to increase the sensitivity of the procedure.

Reactivity of Thiol Groups—The reactivity of the thiol groups of the enzyme was determined by 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent) as described in a previous communication (12).

RESULTS

Modulation of Kinetic and Allosteric Properties of Phosphofructokinase by Ammonium Ions—Fig. 1 shows the response of phosphofructokinase activity to increasing concentrations of its substrate ATP in the absence and in the presence of two different levels of NH$_4^+$. The sensitivity of the enzyme to inhibition by higher concentrations of ATP was apparent in all three curves. As indicated in the insert of Fig. 1, NH$_4^+$ increased the $V_{max}$, lowered the $K_m$ (as calculated from the points below 0.2 mM), and increased the $K_i$ (as calculated from the points above 0.2 mM) with respect to ATP.

Fig. 2 depicts the kinetics of phosphofructokinase with respect to fructose 6-phosphate at an inhibitory level of ATP (0.5 mM). The sigmoidicity was apparent at all NH$_4^+$ concentrations tested. The data summarized in Fig. 2 show that NH$_4^+$ increased the $V_{max}$ and decreased the $K_m$ for fructose 6-phosphate without affecting the Hill number. The high value of the latter (3.7 to 4.2) indicates a high degree of cooperativity among the fructose 6-phosphate binding sites under the conditions used.

Fig. 3 illustrates the effect of NH$_4^+$ on the inhibition of phosphofructokinase by citrate. It is clear from these data that although the enzyme was markedly activated by this cation, it remained sensitive to inhibition by citrate. However, the activator (0.2 mM) raised the $K_i$ for citrate from 0.025 mM to 0.055 mM.

Comparison between Efficiency of NH$_4^+$ and K$^+$ as Activators—Fig. 4 provides a comparison between the activating effect of NH$_4^+$ and K$^+$ ions at an inhibitory concentration of ATP (0.5 mM). It is clear from this graph that NH$_4^+$ ions are much more efficient than K$^+$ ions in activating phosphofructokinase under these simulated physiological conditions. The data in Fig. 4 gave an apparent dissociation constant ($K_d$) of 0.33 mM for the enzyme-NH$_4^+$ complex. This value lies within the physiological range of NH$_4^+$ concentration in several tissues as indicated below.

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ATP is at an inhibitory concentration of 0.5 mM. Other experimental conditions were as described under Fig. 1.

**FIG. 4.** Comparison of NH₄⁺ and K⁺ as activators of phosphofructokinase using inhibitory concentration of ATP (namely, 0.5 mM). The reaction mixture contained 0.5 mM fructose 6-phosphate, 0.3 mM ITP, and 1 mM MgCl₂. Other reactants and conditions were as described under Fig. 1.

**FIG. 5.** Activation of phosphofructokinase (PFK) by NH₄⁺ using ITP as phosphate donor. The reaction mixture contained 0.5 mM fructose 6-phosphate, 0.3 mM ITP, and 1 mM MgCl₂. Other reactants and conditions were as described under Fig. 1.

**FIG. 6.** Activation of phosphofructokinase (PFK) by K⁺ using ITP as phosphate donor. The reaction mixture contained 0.5 mM fructose 6-phosphate, 0.3 mM ITP, and 1 mM MgCl₂. Other reactants and conditions were as described under Fig. 1.

certain metabolites with quaternary ammonium groups, e.g., carnitine, choline, and acetylcholine failed to exert any activating effect on phosphofructokinase. This provides evidence that the binding site for NH₄⁺ on the enzyme is highly specific.

**Rapidity of Binding of NH₄⁺ by Phosphofructokinase—**Ammonia manifests its activating effect on the enzyme quite rapidly. Full activation was obtained within the few seconds necessary for mixing the solution in the test cuvette.

**Reactivity of Thiol Groups of Phosphofructokinase in Presence of NH₄⁺ Ions—**In previous communications (12, 13) we have shown that the reactivity of thiol groups of this enzyme with Ellman's reagent was a sensitive index of conformational changes induced by its modulators. Similar findings were reported by several other workers (14–16). This approach was used in an effort to detect any changes elicited by NH₄⁺. However, we found that ammonium chloride (up to 8 mM) brought about no significant change in the reactivity of thiol groups of phosphofructokinase. In the presence, as well as in the absence of NH₄⁺ about 4 to 6 thiol equivalents (per protomer of 93,000 daltons) reacted very rapidly with Ellman's reagent at pH 8.0, and the remaining 10 to 12 thiol equivalents reacted more slowly. The latter could be revealed rapidly by addition of sodium dodecyl sulfate. Thus it seems that the effect of NH₄⁺ on phosphofructokinase is quite subtle.

**Physiological Concentration of Ammonium Ions—**As indicated above, the apparent dissociation constant of the phosphofructokinase-NH₄⁺ complex in the presence of an inhibitory concentration of ATP was 0.33 mM. In order to determine whether this concentration is physiological, we surveyed the literature for pertinent data. The average concentrations of NH₄⁺ in human erythrocytes (17), rat brain (18), and resting rat leg muscle (19) were found to be 0.24, 0.23, and 0.34 mM, respectively. It is evident from these data that the apparent Kₐ for the enzyme-NH₄⁺ complex is within the physiological range of NH₄⁺ concentration. It is worth noting also that the concentration of NH₄⁺ in the active muscle (1.18 mM) was found to be higher than that in the resting muscle (19).
DISCUSSION

The high specificity of the activation of phosphofructokinase by NH₄⁺ and its action in vitro at concentrations comparable to those in several tissues leads us to ascribe a physiological role to NH₄⁺ in glycolysis. Three systems are known to function in the fixation of ammonia in mammalian systems; these are catalyzed by glutamic dehydrogenase, glutamine synthetase, and carbamyl phosphate synthetase. We noticed that all of these reactions utilize products either of the glycolytic pathway, or of the citric acid cycle, namely, α-ketoglutarate, NADH, ATP, and CO₂ or of all of these. Thus, it seems feasible to assume that the activation of phosphofructokinase by NH₄⁺ enhances the utilization of carbohydrates to produce the compounds essential for its own fixation and the maintenance of its tissue concentrations at tolerable leve’s.

Although the muscle enzyme is activated also by K⁺, the above data indicate the great disparity between the dissociation constants of the two cations, especially at higher concentrations of ATP. In our hands, NH₄⁺ activated phosphofructokinase even in the presence of saturating concentrations of K⁺, as prevails under physiological conditions. Therefore, it seems that the NH₄⁺ site is distinct from the K⁺ site. Related findings were reported for the yeast enzyme by Mavis and Stellwagen (8), and for the Clostridium pasteurianum enzyme by Uyeda and Kurooka (20). Moreover, Kloppick, Jacobasch, and Rappoport (21) have shown that NH₄⁺ stimulated glycolysis in human erythrocytes and the effect was attributed to phosphofructokinase. Their work indicates that NH₄⁺ can stimulate this enzyme in an intact cell endowed with its physiological concentration of K⁺.

The activation of phosphofructokinase by NH₄⁺ has been described as deinhibition of ATP in case of the yeast enzyme (7). The data presented here indicate that such activation is not a mere deinhibition in case of the muscle enzyme. This is borne out by the fact that the activation is observed with ATP, which is a noninhibitory phosphate donor. Although NH₄⁺ increased the K⁺ of ATP, the enzyme remained sensitive to this inhibitory phosphate donor. We have observed a similar effect of NH₄⁺ on the sensitivity of phosphofructokinase to citrate. The fact that a modulator can change the kinetic parameters of the allosteric enzyme without completely reversing its sensitivity to other modifiers is a significant concept in regulation of enzymatic activities. This characteristic would allow the control of an enzymatic activity by several modulators simultaneously, i.e. “collective regulation.”

The data reported here might also have a bearing on the Pasteur effect. Phosphofructokinase has been implicated in the mechanism of this effect by Passonneau and Lowry (22). Since the NH₄⁺ concentration was shown to rise in certain tissues under anoxia (18, 19), it is conceivable that the observed stimulation of glycolysis under these conditions might be attributed, at least in part, to the rise in the NH₄⁺ concentration. Undoubtedly, the levels of adenine nucleotides, inorganic phosphate, and citrate play a role also. Thus the Pasteur effect seems to be the outcome of action of several modulators.

The mode of action of NH₄⁺ on phosphofructokinase remains unknown. The rapidity of activation of the enzyme by this cation and the failure of the rather sensitive method of thiol group reactivity (12) to detect any conformational change point to a subtle structural modification. It is possible that the effects brought about by NH₄⁺ are the results of a minor change in the orientation of orbital of reactive atoms to cause large changes in the velocity of enzymatic reactions.

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