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Communications

Effect of pH on the Kinetics of Frog Muscle Phosphofructokinase*

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SUMMARY

Under appropriate conditions, the activity of phosphofructokinase of skeletal muscle from frog and mouse is extremely sensitive to small changes in pH in the physiological range, a low pH decreasing the affinity of the enzyme for fructose 6-phosphate. It is concluded that shifts in intracellular pH are important in the regulation of phosphofructokinase, but that this effect makes interpretation of data from intact muscle quite difficult.

Recently, many workers have been studying the regulatory function of small molecules that alter enzymatic activity by binding to specific sites on the protein. At the same time, relatively little attention has been given to the physiological importance of other types of changes in the environment. The present communication describes the effect of small differences in pH on phosphofructokinase activity in solutions of rather high ionic strength ($\Gamma/2$ about 0.2). Other actions of pH on glycolytic enzymes in muscle have been described recently (1).

The activity of phosphofructokinase from mammals is also affected by a variety of modifiers. Fructose 6-phosphate, 5'-adenosine monophosphate, inorganic phosphate, fructose-1,6-di-P, and NH_4^+ stimulate (2–4); ATP and citrate inhibit (2, 5, 6).

EXPERIMENTAL PROCEDURE

Phosphofructokinase was obtained from the gastrocnemius muscle of female *Rana pipiens* or from mice of strain C57 BL/6J purchased from Jackson Memorial Laboratories. The muscle was homogenized in 20 mM Tris-acetate buffer (pH 7.5) containing 1 mM EDTA and 2 mM Na_2SO_4 close to 0°. The homogenate was centrifuged for 10 min at 11,000 × *g*. The supernatant fluid was removed and diluted with the same buffer before assay.

The reaction was followed fluorometrically with the use of the method of Passonneau and Lowry (7) in the temperature range from 24–26°. The reaction mixture contained 20 mM imidazole-acetate buffer, 4 mM ATP added as the disodium salt, 5 mM $MgCl_2$, 150 mM Kacetate, 0.016 mM DPNH, 2 mM Na_2SO_4, and and 0.02% serum albumin. Auxiliary enzymes were added as follows: rabbit skeletal muscle aldolase, 0.08 mg per ml; muscle glycerol-phosphate dehydrogenase, 0.02 mg per ml; triosephosphate isomerase, 0.02 mg per ml; and phosphoglucose isomerase, 0.02 mg per ml. Glucose-6-P was added in concentrations varying from 0.125 mM to 2 mM. The fructose-6-P concentration was estimated from the added glucose-6-P, assuming equilibrium of the two hexosephosphates in the presence of phosphoglucose isomerase according to the data of Kahana *et al.* (8). The pH of the reaction mixture was altered by varying the pH of the buffer. The reaction was started by adding muscle extract to a final dilution of 1:50,000 (w/v). After the end of the reaction, the pH of the mixture was measured to the nearest 0.01 unit at 25° on a Radiometer pH meter model 4. The results are expressed as millimicromoles of DPNH produced per min per ml of reaction mixture. Auxiliary enzymes are present in great excess and should not affect the rate at the reaction (7). Altering the concentration of auxiliary enzymes 3-fold did not affect the reaction rate in the range of pH and substrate concentrations reported.

RESULTS AND DISCUSSION

The phosphofructokinase from skeletal muscle of *Rana pipiens* is extremely sensitive to pH under conditions which might be expected to be similar to those found *in vivo*. Fig. 1 illustrates the effect of pH on phosphofructokinase at various concentrations of fructose-6-P in the presence of 0.1 mM 5'-AMP. At 4 mM ATP increase in the concentration of fructose-6-P displaces the pH curve toward the acid side with a simultaneous increase in the phosphofructokinase activity.

Absence of 5'-AMP displaces the curve to the alkaline side. Thus, in Fig. 2, omission of 5'-AMP shifts the midpoint of the curve from 7.0 to 7.12 and from 7.44 to 7.6 at 0.11 mM and 0.05 mM fructose-6-P, respectively. Fig. 3 shows that 5'-AMP has a major stimulatory effect over a very narrow pH range; the actual range, as well as the magnitude of the effect, depends upon the concentration of fructose-6-P. Fig. 4 shows that phosphofructokinase from mammalian muscle also is sensitive to small changes in pH.

To our knowledge, these pH curves are the steepest yet reported for any enzyme, but it would be surprising if physiological regulation of enzyme activity by pH were not a widespread and perhaps primitive method of control.

It is of interest that both the extreme sensitivity to pH and the major stimulatory effect of 5'-AMP are lost when the concentration of ATP is 0.125 mM, a level well below that reported in the frog muscle and also below that at which ATP significantly inhibits phosphofructokinase. Thus, the ATP-inhibited enzyme is in a state most susceptible to these effects. This fact suggests that a rise in pH might either decrease the binding of an active species of ATP to a regulatory site on the enzyme or might antagonize the conformational changes resulting from the binding of ATP to the inhibitory site.

The relationship between pH and the activation of phosphofructokinase by fructose-6-P and 5'-AMP suggests that similar relationships exist in the case of other activators and inhibitors (9, 10). Such interactions give great physiological flexibility and help to account for the extraordinarily effective control of the glycolytic pathway in muscle. Control of glycolysis by pH may also contribute to the regulation of intracellular pH.

The effects reported here may also be used to explain certain phenomena previously noted in intact muscles. Changes in pH do occur within muscle cells (11), and do affect glycolysis (12, 13).

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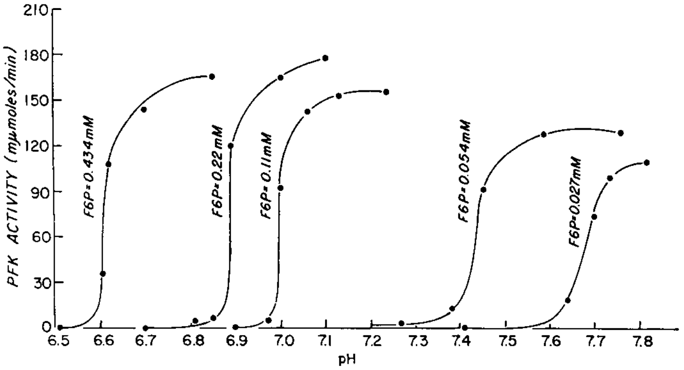

FIG. 1. The effect of pH on phosphofructokinase (*PFK*) activity at various fructose-6-P (*F6P*) concentrations in the presence of 0.1 mM 5′-AMP. The remaining conditions of assay are described in the text.

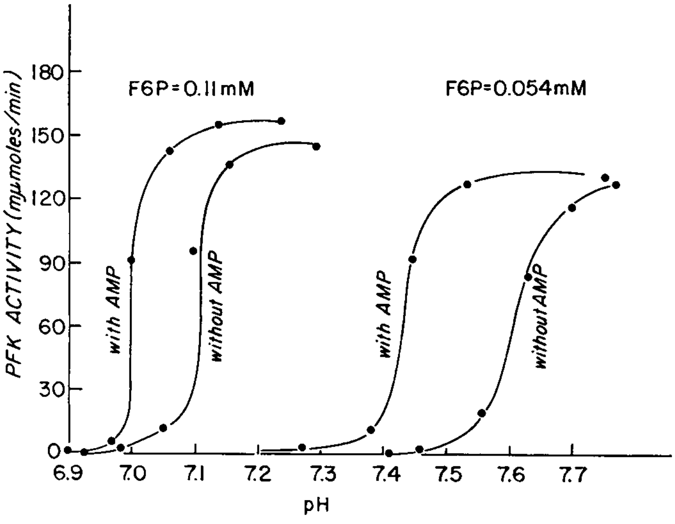

FIG. 2. The effect of 0.1 mM 5′-AMP on the pH activity curve of phosphofructokinase (*PFK*) at two concentrations of fructose-6-P (*F6P*). The conditions of assay are described in the text.

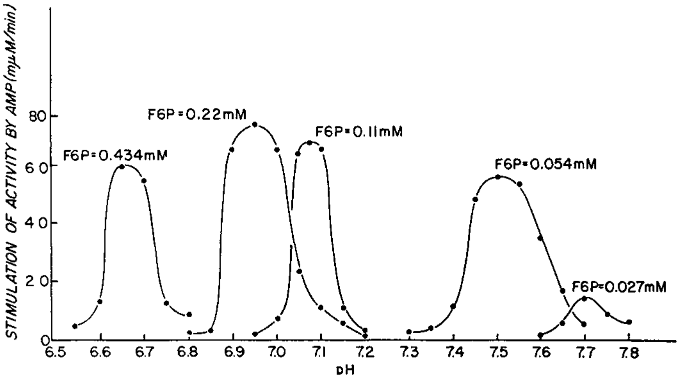

FIG. 3. Stimulation of phosphofructokinase activity by 5′-AMP and its dependence on pH. The data were obtained from graphs such as those plotted in Fig. 2. The activity in the absence of 5′-AMP has been subtracted from the activity in the presence of 0.1 mM 5′-AMP.

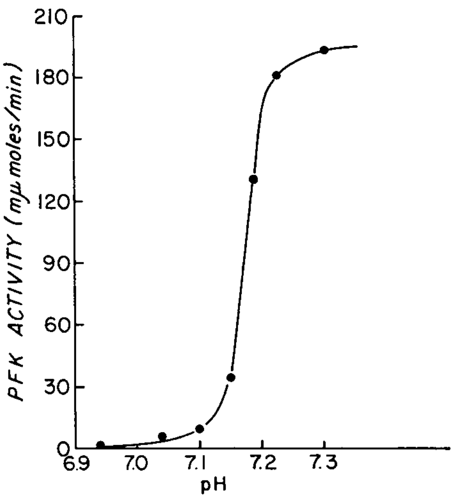

FIG. 4. Effect of pH on phosphofructokinase (*PFK*) from mouse muscle. Fructose-6-P (*F6P*) concentration was 0.22 mM. 5′-AMP was absent from the reaction mixture. Other conditions of the assay are described in the text.

In a series of contractions, muscle first becomes alkaline because of hydrolysis of creatine phosphate (14); at this stage, glycolysis may well be promoted by increased activity of phosphofructokinase. Later, as glycolysis proceeds, the pH falls because of the accumulation of lactic acid (14, 15). A decrease in the apparent affinity of phosphofructokinase for fructose-6-P brought about by this fall in pH probably explains the progressive rise in hexosephosphates which occurs as lactic acid accumulates in contracting frog sartorii (15). It may also account for the fact that lactic acid production stops before the pH falls low enough to damage the cell (1).

However, it is good to note that the situation in intact muscle is quite complex. A simple decrease in affinity of phosphofructokinase for fructose-6-P will not by itself slow glycolysis. When the substrate pool is small compared to the flow over a pathway, a decrease in substrate affinity of one of the enzymes other than the first of the sequence should lead to a progressive rise in substrate concentration. The increasing substrate would, in turn, increase enzyme velocity until the original flow resumed. In order to slow the total flow, other preceding enzymes must also be regulated. Therefore, it is now apparent that an elevation in hexosephosphates does not necessarily indicate that phosphofructokinase has become the rate-limiting enzyme of the glycolytic pathway. One may generalize to say that even a simultaneous rise in substrate and fall in product do not absolutely identify the rate-controlling enzyme. Coordinated changes such as an increase in the K_m value of the enzyme in question and a decrease in the K_m value of the next enzyme in sequence would result in the same pattern. It is, then, very difficult to decide about metabolic control points until detailed information about all the enzymes in a given pathway is available. Conclusions about the rate-limiting steps in the glycolytic pathway made on the basis of changes in concentrations of intermediates should be considered to be tentative rather than final.

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The Nature of the Anthranilic Acid Synthetase Complex of *Escherichia coli**

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SUMMARY

Nonsense mutants with alterations in the E or D gene of the tryptophan operon lack anthranilate synthetase activity. This activity is detected when extracts of D and E mutants are combined. The interacting component in each mutant extract has a low *s* value, while the active species in the mixture has a high *s* value and consists of both components. The *s* value of the complex is the same as the value obtained with wild-type anthranilate synthetase. One of the interacting components is the enzyme catalyzing the succeeding step in tryptophan biosynthesis.

In 1963 Gibson and Jackman (1) reported that chorismic acid is the branch point intermediate in the biosynthesis of aromatic amino acids. Chorismic acid is converted to tryptophan in *Escherichia coli* by enzymes specified by five genes clustered in a small region of the chromosome (2) (Fig. 1).

In the course of studies on the translation of the clustered tryptophan genes, it was observed that some D gene nonsense mutants (mutants with chain-terminating codons) were not only deficient in PR transferase[1] activity, but also lacked anthranilate synthetase activity. Since the E and D genes are distinct genetically, it was thought that the loss of both enzymatic activities as a result of mutations in the D gene might be indicative of some type of interaction between the polypeptides specified by the D and E genes. In this report we show that the protein species responsible for anthranilate synthetase activity is, in fact, a complex of the E gene product and PR transferase, the D gene product.

Mutants E5972 and D9778 are nonsense mutants altered in the E and D genes, respectively. Crude extracts of either mutant alone are incapable of catalyzing the anthranilate synthetase reaction. When the two extracts are mixed, however, activity is observed. Fig. 2 shows that in the presence of a constant amount of D9778 extract, the rate of anthranilate formation from chorismic acid and L-glutamine is proportional to the amount of E5972 extract added. A similar result was obtained with an extract of a deletion mutant which lacks genes A, B, C, and D, and the extract of E5972.

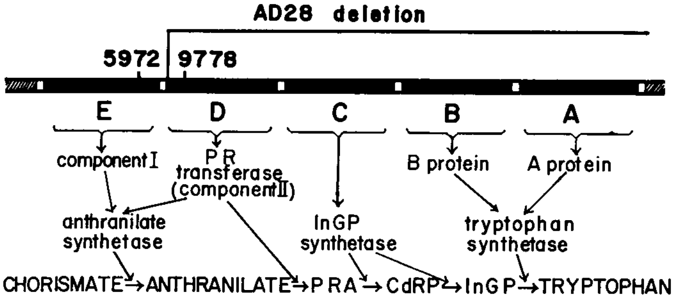

Fig. 1. The tryptophan pathway in *E. coli* and the corresponding genes and enzymes. The positions of the mutationally altered sites in E5972 and D9778 are indicated as well as the extent of the deletion in mutant AD28. *PRA*, *N*-(5′-phosphoribosyl)anthranilate; *CdRP*, 1-(*o*-carboxyphenylamino)-1-deoxyribulose 5-phosphate.

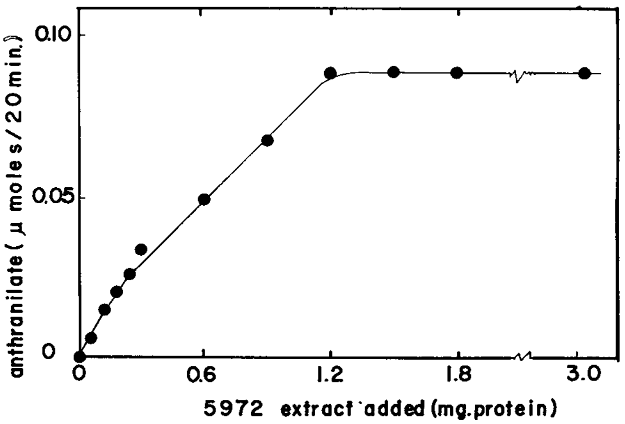

Fig. 2. The influence of an E5972 extract on the formation of anthranilate by a D9778 extract. Anthranilate synthetase activity was assayed as described previously (3). The reaction mixture contained 0.04 ml of D9778 extract (0.3 mg of protein), 2×10^{-4} M chorismic acid, 4×10^{-3} M $MgSO_4$, 1×10^{-2} M L-glutamine, 1×10^{-2} M potassium phosphate buffer at pH 7.6, 1×10^{-3} M β-mercaptoethanol, and the indicated amount of E5972 extract in a final volume of 2 ml.

Direct evidence that a component in the D9778 extract and a component in the E5972 extract combine to constitute the active anthranilate synthetase complex was obtained by zone sedimentation and Sephadex gel filtration studies. Fig. 3*A* shows the sedimentation behavior of the component in D9778 extracts (designated Component I of anthranilate synthetase) which exhibits anthranilate synthetase activity in the presence of an extract of E5972. This component sediments at a rate intermediate between that of the B protein of tryptophan synthetase and InGP synthetase. The $s_{20,w}$ value relative to the B protein of tryptophan synthetase is 4.3 *s*. The sedimentation pattern obtained with extracts of E5972 is shown in Fig. 3*B*. A single component (designated Component II of anthranilate synthetase) active with extracts of D9778 was detected and the activity peak matched the PR transferase activity peak (Fig. 3*B*). The estimated $s_{20,w}$ value was 4.4. When extracts of D9778 and E5972 were simply mixed and the mixture was sedimented in a sucrose gradient, a rapidly sedimenting peak of anthranilate

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[1] The abbreviations used are: PR transferase, anthranilate-5-phosphoribosylpyrophosphate phosphoribosyltransferase; InGP, indole-3-glycerol phosphate.