Interaction between Energy Charge and Metabolite Modulation in the Regulation of Enzymes of Amphibolic Sequences. Phosphofructokinase and Pyruvate Dehydrogenase*

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ABSTRACT: The prediction that energy charge (half the average number of anhydride-bound phosphate groups per adenosine moiety) and the concentration of an indicator metabolite should jointly control the rates of amphibolic sequences (those that contribute both to adenosine triphosphate regeneration and to the supply of primary biosynthetic intermediates) has been tested with rabbit muscle phosphofructokinase and pyruvate dehydrogenase from *Escherichia coli*. Slopes of curves showing the rates of the reactions catalyzed by these enzymes as functions of energy charge are negative in the region above a charge of about 0.6. On the addition of modulating metabolites (citrate for phosphofructokinase; acetyl coenzyme A for pyruvate dehydrogenase), the rates of the reactions at high energy charge are decreased, and the curves of rate against charge become much more steep.

This behavior seems adapted to regulate amphibolic sequences in response to both metabolic demand for primary biosynthetic intermediates and the energy charge of the cell. Adenosine 3′,5′-cyclic monophosphate decreases the response of phosphofructokinase to energy charge. This effect is consistent with the function of the cyclic nucleotide in hormonal interactions that override local cellular control of energy metabolism.

In aerobic cells utilizing carbohydrate, the glycolytic sequence and citrate cycle supply electrons for the regeneration of ATP and also provide primary intermediates needed for the biosynthetic activities of the cell. Davis (1961) proposed the term "amphibolic" for such sequences to emphasize their dual metabolic role. Duality of function requires duality in regulatory mechanism. The first paper of this group (Atkinson, 1968b) points out the advantages that should result if key regulatory enzymes of amphibolic sequences are modulated both by the energy charge (Atkinson and Walton, 1967) and by the concentration of one or more compounds serving as an indicator of the level of primary biosynthetic intermediates.

This paper reports interactions of energy charge with citrate concentration in the modulation of phosphofructokinase activity and with the concentration of *S*-acetyl-CoA[1] in modulating the activity of pyruvate dehydrogenase. An effect of 3′,5′-AMP on the response of phosphofructokinase to variation in energy charge is also reported.

Materials and Methods

Phosphofructokinase. ASSAY. The rate of the reaction was followed either by estimating fructose diphosphate production by coupling to glycerol phosphate dehydrogenase (Ling *et al.*, 1955) or by estimating ADP production with pyruvate kinase and lactic dehydrogenase. The assay mixture in either case contained 100 mM Tris-HCl (pH 8.0), 50 mM KCl, and the appropriate assay enzymes in excess. For the fructose diphosphate assay 100 μM DPNH was added, and for the ADP assay, 200 μM DPNH and 400 μM phosphoenolpyruvate. Disappearance of DPNH was followed with a Gilford spectrophotometer. Assay volume was 1.0 ml.

ENZYMES. Commercial rabbit muscle phosphofructokinase (Boehringer) was dissolved in a buffer solution containing 50 mM Tris-HCl (pH 8.0), 10 mM potassium phosphate, 1 mM dithiothreitol, and bovine serum albumin at about 1 mg/ml. The resulting solution was passed through a Sephadex G-25 column equilibrated with the same buffer.

The accessory enzymes required in the assays (aldolase, triosephosphate isomerase, and glycerol phosphate dehydrogenase in the fructose diphosphate assay, lactic dehydrogenase, and pyruvate kinase in the ADP assay, and adenylate kinase in energy charge experiments) were desalted by passage through a Sephadex G-25 column in 50 mM Tris-HCl (pH 8.0).

Pyruvate Dehydrogenase. ASSAY. The rate of reduction of DPN^+ in the over-all reaction catalyzed by the complex was followed spectrophotometrically at 340 nm (Schwartz *et al.*, 1968). The assay mixture (1.0 ml) contained 50 mM Tris-HCl (pH 7.6), 6 mM $MgCl_2$, 50 μM pyruvate, 65 μM CoASH, 250 μM DPN^+, 500 μM cysteine, and 30 μM thiamine pyrophosphate. The reaction was initiated by addition of enzyme complex (about 1 μg of protein).

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[1] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: *S*-acetyl-CoA, *S*-acetyl coenzyme A.

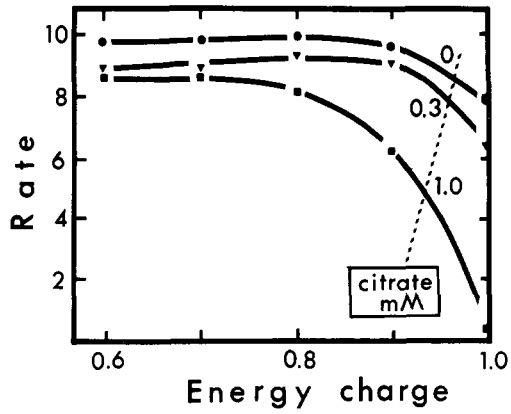

FIGURE 1: Rate of the reaction catalyzed by rabbit muscle phosphofructokinase as a function of energy charge; effect of citrate. Mixtures of ATP and AMP of the desired energy charge and at a combined concentration of 6 mM were added to the components of the ADP assay for phosphofructokinase (coupled to lactate dehydrogenase). The concentration of fructose diphosphate was 0.5 mM, and Mg^{2+} concentration was 6 mM. Citrate was added as indicated.

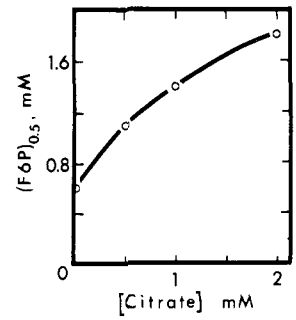

FIGURE 2: Effect of citrate on the concentration of fructose 6-phosphate required for half-maximal velocity of the reaction catalyzed by rabbit muscle phosphofructokinase. A 9:1 mixture of ATP and AMP (0.9 charge; total adenylate concentration in assay mixture, 6 mM) was incubated with excess adenylate kinase and $MgCl_2$ (concentration in assay, 6 mM) for 15 min before addition of the components of the fructose 6-phosphate assay for phosphofructokinase (coupled to α-glycerophosphate dehydrogenase). Values of $(F\text{-}6\text{-}P)_{0.5}$ were estimated from Hill plots, using values of V_{max} derived from double-reciprocal (Lineweaver–Burk) plots.

PREPARATION OF COMPLEX. The pyruvate dehydrogenase complex was isolated from *Escherichia coli*, Crookes strain, grown in medium containing 90 mM sodium pyruvate, 15 mM sodium glutamate, 5 mg/l. of yeast extract, and mineral salts. The complex was purified about 30-fold by protamine sulfate precipitation and elution, as described by Koike *et al.* (1963), and stored in 50 mM Tris-HCl (pH 7.0) containing 1 mg of bovine serum albumin/ml.

Results

Phosphofructokinase. INTERACTION BETWEEN ENERGY CHARGE AND CITRATE FEEDBACK INHIBITION. The primary effect of ATP as a negative modifier for phosphofructokinase is to increase $(F\text{-}6\text{-}P)_{0.5}$ (decrease the affinity of the enzyme for fructose 6-phosphate). This effect is counteracted by AMP. Thus the sharpness of the response of phosphofructokinase to variation in energy charge will depend upon the concentration of fructose 6-phosphate; the inhibitory effect of high energy charge will be most pronounced when the concentration of substrate is low. At 0.5 mM fructose 6-phosphate, the effect of variation in energy charge is small (Figure 1). The response changes strikingly on the addition of citrate, a negative modifier for phosphofructokinase (Parmeggiani and Bowman, 1963; Passonneau and Lowry, 1963; Garland *et al.*, 1963). Under standard assay conditions ATP, but not ADP or AMP, is present; thus the previously reported effects of citrate were observed at an energy charge of 1.0. The results presented in Figure 1 show that, in addition to inhibiting at a charge of 1.0, citrate strongly increases the steepness of response to energy charge in the presumed physiological range around 0.8–0.9 charge. Salas *et al.* (1965) reported the related observation that citrate appeared to increase the sensitivity to ATP of yeast phosphofructokinase.

At a fixed energy charge, the affinity of the enzyme for fructose 6-phosphate decreases on the addition of citrate, as illustrated for a charge of 0.9 in Figure 2. The result does not, of course, indicate whether the effect on fructose 6-phosphate binding is exerted directly or indirectly, for example, by changing the affinity of the regulatory site for ATP or AMP, or by altering the interactions between the regulatory and catalytic sites.

INTERACTION BETWEEN ENERGY CHARGE AND CYCLIC AMP. Cyclic 3′,5′-AMP is a positive modifier for phosphofructokinases from the liver fluke *Fasciola hepatica* (Mansour and Mansour, 1962) and from mammalian sources (Passonneau and Lowry, 1962; Mansour, 1963). The effect of this nucleotide on the response of rabbit muscle phosphofructokinase to energy charge is shown in Figure 3. Under the conditions of this experiment (0.25 mM fructose 6-phosphate) the reaction is inhibited strongly by high energy charge, and this response is much less pronounced when 3′,5′-AMP is added. The results observed at energy charges of 0.8, 0.9, and 1.0 are replotted as functions of the concentration of cyclic AMP in the insert of Figure 3.

Pyruvate Dehydrogenase. The pyruvate dehydrogenase complex from pig heart (Garland and Randle, 1964) and *E. coli* (Hansen and Henning, 1966) is inhibited by *S*-acetyl-CoA. Schwartz and Reed (1968) have reported that the activity of the enzyme complex is affected also by nucleotides.

Figure 4 shows the rate of DPNH production (which requires the action of all three enzymes of the complex) as a function of energy charge and the concentration of *S*-acetyl-CoA. The effect of energy charge alone is small, but the curves of rate as a function of energy charge become much steeper when *S*-acetyl-CoA is added.

Schwartz *et al.* (1968) have recently reported that the inhibitory effect of *S*-acetyl-CoA on the complex is exerted on the enzyme catalyzing the first reaction. We have confirmed this observation. This same enzyme (pyruvate dehydrogenase) also appears to be the main

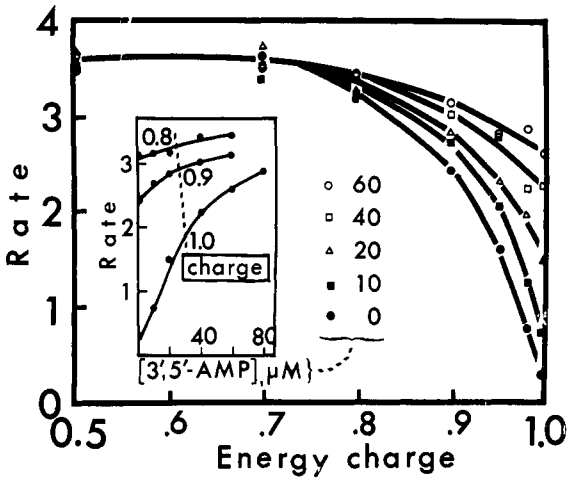

FIGURE 3: Rate of the reaction catalyzed by rabbit muscle phosphofructokinase as a function of energy charge; effect of 3′,5′-AMP. Mixtures of AMP and ATP at the desired energy charge (total andenylate concentration in assay mixture, 4 mM) were incubated with excess adenylate kinase and $MgCl_2$ (concentration in assay, 2 mM) before addition of the components of the fructose diphosphate assay for phosphofructokinase. The concentration of fructose 6-phosphate was 0.25 mM, and 3′,5′-AMP was added as indicated. Insert: replot of the results obtained at charge values of 0.8, 0.9, and 1.0 as functions of the concentration of 3′,5′-AMP.

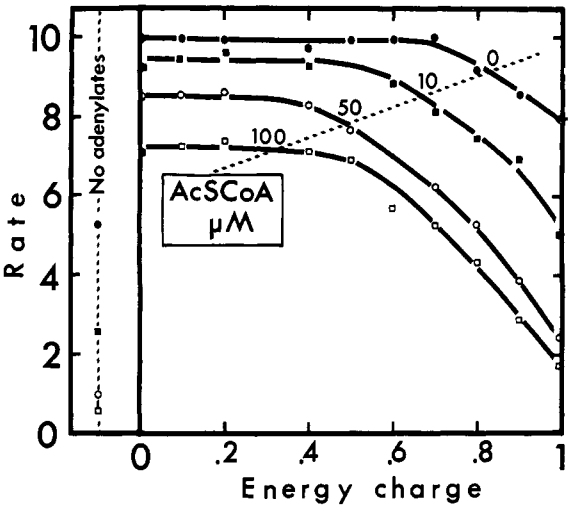

FIGURE 4: Rate of the reaction sequence catalyzed by the *E. coli* pyruvate dehydrogenase complex: effect of acetyl-SCoA. The assay method is described in a previous section. Desired values of energy charge were established by addition of appropriate amounts of AMP, ADP, and ATP, calculated from an equilibrium constant for the adenylate kinase reaction of 0.8. The total adenylate concentration was 5 mM. S-Acetyl-CoA was added as indicated. Rates observed in the absence of adenine nucleotides are indicated at the left side of the graph.

site of modulation by energy charge. When pyruvate dehydrogenase was dissociated from the complex (Koike *et al.*, 1963) and assayed by measuring CO_2 evolution from pyruvate-1-^{14}C, the response pattern was similar to that seen in Figure 4.

Discussion

Both phosphofructokinase and pyruvate dehydrogenase respond to energy charge and to the concentration of a feedback metabolite in the way that seems appropriate for a regulatory enzyme in an amphibolic sequence (Atkinson, 1968b). The response to high energy charge in the absence of the feedback metabolite is small, as is the response to metabolite concentration when the energy charge is low. An increase in the value of either regulatory parameter enhances the effect of the other. Thus the two functions of the amphibolic sequence are reflected in its control, and the rate of the sequence should be relatively rapid when either the energy charge or the concentration of the modulating metabolite is below its usual range. Under normal physiological conditions, the rate of the sequence should be simultaneously a sensitive function of both control parameters.

Phosphofructokinase. Passonneau and Lowry (1962) suggested that the oppositely directed effects of ATP and AMP on phosphofructokinase might contribute importantly to the genesis of the Pasteur effect. These effects were also among the facts that led to the broader suggestions that variations in the concentration of AMP may play an important role in the metabolic choice between glycolysis and gluconeogenesis (Krebs, 1964) and that the balance among the concentrations of AMP, ADP, and ATP may be a primary determinant of the rate and direction of metabolic sequences generally (Ramaiah *et al.*, 1964; Atkinson, 1965, 1966).

These suggestions have been questioned on various grounds. Betz and Moore (1967), on the basis of work with yeast extracts, feel that the level of AMP is unlikely to become high enough in an intact cell to lead to any significant effects on phosphofructokinase. The results presented in Figures 1 and 3 indicate that, with reactants and nucleotides at reasonably physiological concentrations, the enzyme responds to quite small concentrations of AMP. When the charge changes from 1.0 to 0.9, the percentage change in the concentration of ATP is relatively small; the response of phosphofructokinase to energy charge in this range depends mainly upon the variation in AMP concentration. With a 4 mM adenylate pool (Figure 3), the concentration of AMP will be 0 at a charge of 1.0, about 25 μM at 0.95, and about 100 μM at 0.90. For a 6 mM adenylate pool (Figure 1), corresponding approximate values are 40 and 150 μM. Under conditions that lead to sharp responses like those illustrated by the control curve of Figure 3 and the 1.0 mM citrate curve of Figure 1, it is clear that (a) the absolute level of AMP required for a significant effect on phosphofructokinase behavior is very low, and (b) in the steep region of the curve, a small percentage change in the concentration of AMP (and a still smaller percentage change in the energy charge) will affect the enzyme significantly.

The significance of adenylate control has also been questioned because it is felt that the concentrations of adenine nucleotides do not vary enough in the intact cell to serve effectively as regulatory parameters. As discussed in the preceding paragraph, quite small changes in concentrations of individual nucleotides, or

in energy charge, may significantly affect the behavior of phosphofructokinase. More importantly, constancy of nucleotide concentrations must have a metabolic cause. We have suggested (Atkinson and Walton, 1967; Atkinson, 1968a,b) that the intersection of steeply falling curves like those in Figures 1 and 3 with steeply rising curves (representing ATP-utilizing reactions) may graphically represent the type of interaction between ATP-regenerating and ATP-utilizing sequences that underlies metabolic regulation. If this suggestion is valid, the relative constancy of the concentrations of the adenine nucleotides is a *consequence* of adenylate control of individual enzymes. The observation that the levels of these nucleotides are nearly constant clearly cannot be considered to be proof of the validity of the adenylate control hypothesis (especially since the hypothesis is in large part an attempt to explain such constancy); on the other hand, it is equally clear that the truth of this prediction of the hypothesis cannot be considered to invalidate it. To suggest a crude analogy, fluctuations in the output voltage of a good voltage regulator are small, but it is nevertheless these fluctuations to which the regulatory elements of the device respond.

Effect of cyclic AMP. Epinephrine, and perhaps hormones generally, may be viewed as a component of an override mechanism by which local control is partially superseded by a signal reflecting organism-wide needs. A major effect of epinephrine on energy metabolism in muscle is to override the energy charge control mechanisms by which the tissue ordinarily regulates its consumption of substrate. Cyclic AMP is the "second messenger" (Sutherland and Robison, 1966) through which some or all of the effects of epinephrine in muscle are exerted.

The first energy control point in carbohydrate metabolism is the phosphorolysis of polysaccharide. Here cyclic AMP leads to evasion of energy charge control by the series of events that culminate in the conversion of phosphorylase *b* into phosphorylase *a*. The second demonstrated energy charge control point is the reaction catalyzed by phosphofructokinase. In contrast to the complexity of the phosphorylase regulatory system, the cyclic nucleotide seems to act directly on phosphofructokinase. The result is functionally similar—the enzyme is released from control by local energy charge. This point is shown by Figure 3, and especially by the insert plot of that figure. The cyclic nucleotide does not activate phosphofructokinase generally, but specifically releases the enzyme from energy charge control. These results and interpretation are compatible with the early observation that 3′,5′-AMP renders phosphofructokinase less susceptible to inhibition by ATP (Mansour, 1963).

Pyruvate Dehydrogenase Complex. The results presented in Figure 4 illustrate some of the complexities that may be met in considering interaction between enzymes and their modifiers. In this case the feedback metabolite, *S*-acetyl-CoA, inhibits very strongly in the absence of nucleotides. Each of the adenine nucleotides, when tested singly, causes an increase in rate, whether or not *S*-acetyl-CoA is also present. The fact that even ATP stimulates, although much less strongly than AMP, may seem inconsistent with the adenylate control hypothesis. Absence of adenine nucleotides is a totally nonphysiological situation, however, and the range from no adenine nucleotides through increasing concentrations of any of them is not an input variable to which a regulatory response has evolved. In the context of a constant adenylate pool, the enzyme is seen to respond, as expected, by a decrease in activity at high energy charge (Figure 4); thus in terms of probable *in vivo* function it appears more appropriate to consider ATP to be inhibitory, rather than stimulatory.

Interactions between an enzyme and another single species of molecule or ion may be of interest for many reasons, including an attempt to elucidate the structural basis underlying the behavior of regulatory enzymes. Where metabolic regulation is the subject of interest, however, it seems desirable that suggestions should be based only on responses of the enzyme to physiologically realistic variables. Proposal of the energy charge was an attempt to supply such a variable for use in study of adenylate control. Parameters analogous to energy charge should be useful in the study of other aspects of metabolism. It is evident, for example, that the mole fraction of TPNH in the TPNH–TPN^+ system and of DPNH in the DPNH–DPN^+ system are more realistic variables in this sense than is the concentration of any single pyridine nucleotide.

The usefulness of the energy charge concept in the interpretation of modifier effects is illustrated by the marked responses to variation in charge (in the physiologically meaningful direction in each case) that are exhibited by yeast citrate synthetase (Atkinson, 1968a) and by the *E. coli* pyruvate dehydrogenase complex in the presence of *S*-acetyl-CoA (Figure 4). Each of the adenine nucleotides, when tested alone, is found to inhibit citrate synthetase (Hathaway and Atkinson, 1965) and to stimulate pyruvate dehydrogenase; these individual effects do not supply a satisfactory basis for proposals regarding regulatory function.

Other metabolite modulators will doubtless modify the pattern seen in Figure 4, but such modulators should be studied in terms of their effects on this already-demonstrated interaction between energy charge and *S*-acetyl-CoA rather than in isolation. On the basis of results presented here and in the preceding paper (Klungsøyr *et al.*, 1968), we suggest that tests of the effect of a suspected enzyme modifier should always include experiments in which energy charge is a primary variable.

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Effect of Alkylguanidines and Alkylamines on Trypsin Catalysis*

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ABSTRACT: A series of alkylguanidine hydrochlorides has been prepared, and their inhibitory action against trypsin catalysis has been studied. Inhibition constants obtained have indicated alkylguanidines are stronger inhibitors than the corresponding amines. Methyl- and ethylguanidines increase the rate of the hydrolysis of acetylglycine ethyl ester catalyzed by trypsin seven- and twofold, respectively. However, the extents of the activation are less than that obtainable with ethylamine. The dissociation constant of the methylguanidine–trypsin complex in the activation is identical with the inhibition constant, indicating that binding of the recognition site of enzyme causes the activation. The activation of the acetylglycine ethyl ester hydrolysis by methylguanidine and ethylamine has been studied as a function of temperature and pH. Results have been obtained which indicate that a shift of the pH–activity profile to a lower pH is a factor contributing to the apparent increase in the rate in the activated catalysis. However, the major factor causing the increased rate seems to be an increase in the entropy of activation and not a decrease in the heat of activation, as suggested by a temperature independence of the relative increment of the rate of the acetylglycine ethyl ester hydrolysis in the activation experiment using methylguanidine and ethylamine as activators.

It has been found that trypsin is inhibited by alkylamines at pH 6.6 (Inagami, 1964). Trypsin has also been found to catalyze the hydrolysis of a nonspecific substrate Ac-Gly-OEt[1] (Inagami and Mitsuda, 1964). The rate of this nonspecific catalysis is increased considerably by methyl- and ethylamines whereas their higher homologs, 1-propyl-, and *n*-butylamines, inhibit even the nonspecific catalysis of the Ac-Gly-OEt hydrolysis (Inagami and Murachi, 1964). The activation by the methyl-, and ethylamines has been considered as the indication of the role played by the basic side chain of a specific substrate of trypsin. However, the maximum rate obtained by such an activation was considerably

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Ac-Gly-OEt, acetylglycine ethyl ester; Bz-Arg-OEt, α-*N*-benzoyl-L-arginine ethyl ester.