Regulation at the Phosphoenolpyruvate Branchpoint by the Adenylate Energy Charge in *Mytilus edulis*

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Pyruvate kinase (EC 2.7.1.40) is an enzyme with high activity in most tissues. Phosphoenolpyruvate carboxykinase (EC 4.1.1.32) always has a restricted distribution. In vertebrates phosphoenolpyruvate carboxykinase is found in tissues, such as liver and kidney cortex, with high gluconeogenic activities (Scrutton & Utter, 1968). In these tissues the enzyme operates together with pyruvate carboxylase in the 'dicarboxylic acid shuttle', which serves to generate phosphoenolpyruvate from non-carbohydrate sources under metabolic conditions in which the net flux of the Embden–Meyerhof pathway is towards glucose. Similarly, in many bacteria phosphoenolpyruvate carboxykinase is linked to biosynthetic pathways and serves, by the production of oxalacetate, to replace the tricarboxylic acid-cycle intermediates that are consumed in biosynthetic sequences (Liao & Atkinson, 1971a). In many invertebrates, however, phosphoenolpyruvate carboxykinase participates in degradative sequences and the enzyme is found with high activity in glycolytic tissues. This is especially true for the sea mussel *Mytilus edulis* in which the posterior adductor muscle shows the highest activity of all the body tissues (de Zwaan, 1972). Its role is to convert phosphoenolpyruvate into oxaloacetate, which in turn is converted into the glycolytic end products. Whatever the fate of phosphoenolpyruvate, conversion into glucose or into aerobic or anaerobic end products, the phosphoenolpyruvate branchpoint must be regulated when both pyruvate kinase and phosphoenolpyruvate carboxykinase are present in the same tissue or cell. It appears that in such tissues (liver, kidney cortex, posterior adductor muscle) as well as in bacterial species, pyruvate kinase shows allosteric behaviour and therefore is affected by many modulators (Llorente et al., 1970; Liao & Atkinson, 1971a,b; de Zwaan & Holwerda, 1972; Holwerda & de Zwaan, 1973).

Atkinson (1968) postulated that at any point where a metabolite is partitioned between energy-yielding (degradative sequences) and energy-demanding (biosynthetic sequences) processes the energy charge ([ATP]+1/2[ADP])/([ATP]+[ADP]+[AMP]) is a major regulatory factor. As far as the phosphoenolpyruvate branchpoint is concerned *Azoto bacter vinelandii* is the only example known by us in which the two competing enzymes phosphoenolpyruvate carboxylase (EC 4.1.1.31) and pyruvate kinase from one species or tissue have been studied in relation to the energy charge. In this species, pyruvate kinase is activated by a decrease in the energy charge, whereas phosphoenolpyruvate carboxylase is not under (direct) control of this parameter. The last result is understandable, as none of the compounds that determine the energy-charge value participate in the reaction:

\[
\text{Phosphoenolpyruvate} + \text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{oxaloacetate} + \text{P}_i
\]

When this step is catalysed by phosphoenolpyruvate carboxykinase, IDP (or GDP) and ITP (or GTP) are reactants:

\[
\text{IDP} + \text{phosphoenolpyruvate} + \text{CO}_2 \leftrightarrow \text{ITP} + \text{oxaloacetate}
\]

In this case we may expect a direct effect on enzyme activity, because the various nucleoside phosphate energy charges are kept in equilibrium with the adenylate energy charge by the action of nucleoside diphosphate kinase (EC 2.7.4.6.) (Thompson & Atkinson, 1971). According to Atkinson (1968) a U-type response can be predicted when the enzyme acts in the dicarboxylic acid cycle (liver, kidney cortex) and an R-type response when the enzyme catalyses a step in the overall anaerobic glycolytic pathway (adductor muscle).
This paper deals with a study of the effect of the energy charge on the activities of pyruvate kinase and phosphoenolpyruvate carboxykinase from the posterior adductor muscle of *M. edulis*. Both enzymes are known to catalyse a step in a degradative sequence; during anaerobiosis (shell closure) phosphoenolpyruvate carboxykinase predominates and during aerobiosis pyruvate kinase predominates. Other papers dealing with the anaerobic-aerobic transition at the phosphoenolpyruvate level in adductor muscle have appeared in which the effects of individual nucleoside phosphates on both enzymes are described (Mustafa & Hochachka, 1971; de Zwaan, 1972; de Zwaan & Holwerda, 1972; Mustafa & Hochachka, 1973a,b; Holwerda & de Zwaan, 1973; de Zwaan & de Bont, 1975). In living material, which always contains adenylate kinase, all three adenylates (AMP, ADP and ATP) will be present in fixed proportions at the same time. These proportions determine the energy-charge value and therefore the use of this parameter is a better approach from a physiological point of view.

Sea mussels (*M. edulis*) were taken from the same stock as used in the previous study (Wijman et al., 1976). The enzyme preparation and the assay procedure for pyruvate kinase were the same as described by de Zwaan (1972). All experiments were carried out in 100 mM-imidazole buffer (pH values as indicated in Fig. 1), 67 mM-KCl, 1.5 mM-Mg$^{2+}$ (free ion), 0.12 mM-NADH, excess of lactate dehydrogenase, 0.2 mM-phosphoenolpyruvate, total adenylate concentration (AMP+ADP+ATP) 5 mM. The enzyme preparation and assay procedure for phosphoenolpyruvate carboxykinase were the same as described by de Zwaan & de Bont (1975). All experiments were carried out in 50 mM-KH$_2$PO$_4$/K$_2$HPO$_4$ buffer, pH 6.65, 0.5 mM-Zn$^{2+}$, 1.5 mM-Mg$^{2+}$ (free ion), 10 mM-KHCO$_3$, 0.12 mM-NADH, excess of malate dehydrogenase, 0.2 mM-phosphoenolpyruvate, total adenylate concentration 5 mM, total inosinylate concentration (ITP+IDP) 1 mM.

The concentration of free Mg$^{2+}$ will rise as the energy charge falls and this also could be responsible (in part) for the results obtained. Therefore we have used for both enzymes a magnesium buffer, described by Raaflaub (1956) (100 mM-magnesium citrate/potassium citrate, pH 2.82, as well as the normal pH buffers. The desired energy-charge values were obtained by incubating calculated concentrations of AMP and ATP with excess of adenylate kinase (EC 2.7.4.3). For phosphoenolpyruvate carboxykinase, nucleoside diphosphate kinase was also added to the cuvette, and IDP and ITP were added to the reaction mixture.

![Plot of the initial velocity of the adductor-muscle pyruvate kinase reaction of M. edulis versus energy charge at various pH values](image-url)

*Fig. 1. Plot of the initial velocity of the adductor-muscle pyruvate kinase reaction of M. edulis versus energy charge at various pH values.*

The range of regulation indicates the zone within which the physiological values change during anaerobiosis.
added in a ratio equal to ADP and ATP. Na⁺ were avoided as counterions because Na⁺ causes a marked decrease in enzyme activity. The amount of extract used was always in the range where rates were directly proportional to the amount of protein present.

Fig. 1 shows the plots of enzyme activity against the energy charge for pyruvate kinase at various pH values and Fig. 2 shows the same curve for phosphoenolpyruvate carboxykinase at pH 6.65. Both enzymes respond sharply to changes in the energy charge in the region above 0.6. All curves are concave downwards and therefore belong to the R type classification of Atkinson (1968), which indicates enzymes that catalyse reactions participating in ATP-regenerating sequences.

It has been shown in our laboratory that after 3 days of anaerobiosis the adenylate energy charge decreases from 0.91 to 0.67 (Wijsman et al., 1976). The results in the present paper show that in this region both enzymes are strongly affected by small changes in the energy charge. The observation that both enzymes react to a fall in the energy charge by increasing their catalytic activity to the same extent shows that this factor cannot be responsible for the switch towards the phosphoenolpyruvate carboxykinase during anoxia. Kinetic studies have revealed that both enzymes are oppositely sensitive to changes in pH (de Zwaan & de Bont, 1975). In the sea mussel the pH varies between 7.5 and 6.5 and anaerobiosis causes a shift towards low pH values (Wijsman, 1975). Fig. 1 shows that the shape of the energy-charge-versus-velocity curve does not depend on pH; for all tested pH values the enzyme activity increases to about 160% of its original value when the energy charge falls from 0.91 to 0.67. The absolute activities are strongly affected by pH so that the lower the pH the lower the enzyme activity at any given value of energy charge. During anaerobiosis there will be a simultaneous fall in pH (from 7.5 to 6.5) and energy charge (from 0.91 to 0.67). This will result in an overall decrease in enzyme activity to 53% of its 'aerobic' value (pH 7.5 and energy charge 0.91). For phosphoenolpyruvate carboxykinase a fall in the pH from 7.5 to 6.5 will result in an activation of the enzyme (de Zwaan & de Bont, 1975), and, in this case, pH acts in the same direction as a fall in energy charge. Assuming that both responses are independent, there will be an additive activation effect. The findings presented in this report indicate that the energy charge acting as a regulatory factor on the phosphoenolpyruvate branchpoint is less important than pH. Owing to a fall in the energy charge during anaerobiosis, the total
flux of substrate through the phosphoenolpyruvate pathway, which is the main degradative route under this condition, will increase. The result for *M. edulis* differs from that obtained for *A. vinelandii* as far as the phosphoenolpyruvate → oxaloacetate step is concerned, and this difference fits very well with the role that this step plays in both organisms.


Some Kinetic and Regulatory Properties of the Cytoplasmic L-Malate Dehydrogenases from the Posterior Adductor Muscle and Mantle Tissues of the Common Mussel *Mytilus edulis*

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The anaerobic metabolism of the common mussel *Mytilus edulis* is different from that of vertebrate skeletal muscle. Instead of causing an accumulation of lactate, phosphoenolpyruvate is converted into either pyruvate (and probably then into alanine) by pyruvate kinase or oxaloacetate by cytoplasmic phosphoenolpyruvate carboxykinase. Oxaloacetate is reduced to malate in the cytoplasm by L-malate dehydrogenase (EC 1.1.1.37), and malate (or fumarate) enters the mitochondrion and is reduced to succinate. Succinate is probably converted into propionate [for a review see de Zwaan et al. (1976)]. The phosphoenolpyruvate carboxykinase reaction is thought to predominate over the pyruvate kinase reaction with increasing anaerobiosis (de Zwaan & van Marrewijk, 1973; Livingstone & Bayne, 1974; Kluytmans et al., 1975), and it is hypothesized that, by this metabolic scheme, the ATP yield per molecule of glucose 6-phosphate is increased (de Zwaan et al., 1976; de Zwaan & Wijisman, 1976). The reduction of oxaloacetate to malate is a key reaction in the anaerobic pathway, and it is generally thought that in bivalves such as *Mytilus*, malate dehydrogenase replaces lactate dehydrogenase in re-oxidizing cytoplasmic NADH (generated anoxically). The ratios of malate dehydrogenase/lactate dehydrogenase are always high in organisms forming succinate (see de Zwaan et al., 1976). A study of malate dehydrogenase from two functionally different tissues of *Mytilus* was considered important for several reasons: (1) malate accumulation occurs in the posterior adductor muscle under certain conditions of hypoxia; (2) a more acute response of the posterior adductor muscle, compared with the mantle, to anaerobiosis has been indicated (D. R. Livingstone & B. L. Bayne, unpublished work); (3) it has been shown that bovine heart cytoplasmic malate dehydrogenase exists in an allosteric form which is inhibited by n-fructose 1,6-bisphosphate (Cassman, 1973; Cassman & Vetterlein, 1974; Vetterlein & Cassman, 1974).