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Stabilization of Adenylate Energy Charge by the Adenylate Deaminase Reaction*

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SUMMARY

In the physiological range of the adenylate energy charge in liver (0.7 to 0.9) the rate of deamination of AMP catalyzed by rat liver adenylate deaminase (EC 3.5.4.6) increases sharply with decreasing energy charge. It is suggested that this response serves to protect against sharp transient decreases in energy charge: when the charge decreases the resulting removal of AMP by deamination will oppose the decrease in charge (the mole fraction of ATP plus half the mole fraction of ADP). The activity of the enzyme decreases sharply as the size of the adenine nucleotide pool decreases in and below the physiological range. This effect may be a self-limiting response to prevent excessive depletion of the pool. These suggestions, based on the properties of the enzyme observed *in vitro*, are consistent with the results of experiments on liver *in vivo* reported by other workers.

In liver, as in most other tissues and organisms studied, the adenylate energy charge (the mole fraction of ATP plus half the mole fraction of ADP (1)) has been shown to be maintained at a value of approximately 0.90 (Refs. 2 and 3; compilation in Ref. 4). When liver is subjected to metabolic stress, such as the trapping of large amounts of phosphate that results from the presence of abnormally high levels of a phosphoryl acceptor, a relatively small drop in the energy charge value is accompanied by a decrease in the total concentration of adenine nucleotides (5–8). This is illustrated by a very interesting experiment reported by Raivio *et al.* (7), whose results are plotted in Fig. 1. Rats injected with fructose exhibit a rapid decrease in hepatic adenine nucleotide concentration. Krebs and co-workers (8) obtained similar results using a perfused liver system, and showed that the decrease in the adenine nucleotide level was accompanied by an increase in the concentration of IMP. This result suggests that the decrease in the adenylate pool level may result from the action of adenylate deaminase. If this enzyme were to become active when the charge falls, the resulting removal of AMP would tend to buffer or protect the value of the energy charge (since removal of AMP must increase the mole fractions of ATP and ADP). This protection would be, of course, at the expense of a decrease in the total adenylate pool.

The proposed protection requires that the adenylate deaminase reaction go slowly, if at all, under normal physiological conditions, but more rapidly as the charge falls. The reported stimulation of the liver enzyme by ATP (9, 10) seemed to argue against such a response, because the level of ATP decreases with decreasing energy charge. Nevertheless, it seemed of interest to study the pattern of response of adenylate deaminase to changes in energy charge and adenylate pool size *in vitro*, with concentrations of substrate and effectors chosen to resemble those to be expected in the intact cell.

A regulatory role, somewhat different from that suggested here, was proposed by Lee and Wang (11) for the effects of ATP and phosphate on the activity of adenylate deaminase. Woods *et al.* (8) suggested that the release of inhibition of adenylate deaminase when the phosphate level falls contributes to the decrease in adenylate pool level in fructose-perfused rat liver.

Adenylate deaminase activity was assayed by coupling the product ammonia to the glutamate dehydrogenase reaction, as described in the legend of Fig. 2. This assay duplicated the results obtained using the standard assay for AMP deaminase, in which the conversion of AMP to IMP is followed by the change in absorption at 285 nm (12). The glutamate dehydrogenase-coupled assay is about 25 times as sensitive as the standard assay, and is not restricted to the low adenine nucleotide concentrations that are feasible for direct spectrophotometric determination.

Liver adenylate deaminase was activated by ATP under all conditions examined. Fig. 2 shows the velocity of the reaction as a function of AMP concentration in the absence and presence of 2 mM ATP. The *inset* shows that at physiological AMP concentrations (0.1 to 0.3 mM), ATP increases the rate of the reaction up to 100-fold. The effect is largely on the affinity of the enzyme for substrate; $S_{0.5}$ values for AMP are 11.1 and 0.3 mM in the absence and presence of ATP, respectively.[1] The corresponding Hill coefficients are 1.7 and 1.1. Activation of adenylate deaminase requires Mg^{2+}. At an AMP concentration of 0.2 mM, $M_{0.5}$ for activation by MgATP is 0.7 mM. The reaction is also activated by ADP, but to a lesser degree.

When the sum of the adenine nucleotides is kept constant at 4 mM, which is close to the level normally found in liver, adenylate deaminase responds to variation in the energy charge as shown by the *upper curve* in Fig. 3. The *lower curve* shows the rate of the reaction under identical conditions, except that ADP and ATP were omitted.

The shape of the energy charge response curve illustrates that, although the enzyme is activated by ATP, the reaction velocity is nevertheless maximal at a low value of energy charge (0.6 to 0.7) and proceeds much more slowly at physiological energy charge (0.9) because the concentration of substrate AMP is limiting under the latter conditions. This behavior is consistent with the suggested role of the enzyme in buffering against large fluctuations in the value of energy charge. Furthermore, the rate of the reaction, in the presence of all three adenine nucleotides, is most sensitive to variation in the energy charge precisely n the range of energy charge values (0.9 to 0.7) that have been

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[1] The symbols used are: $S_{0.5}$, concentration of substrate required for half-maximal reaction velocity; $M_{0.5}$, concentration of modifier required for half-maximal effect.

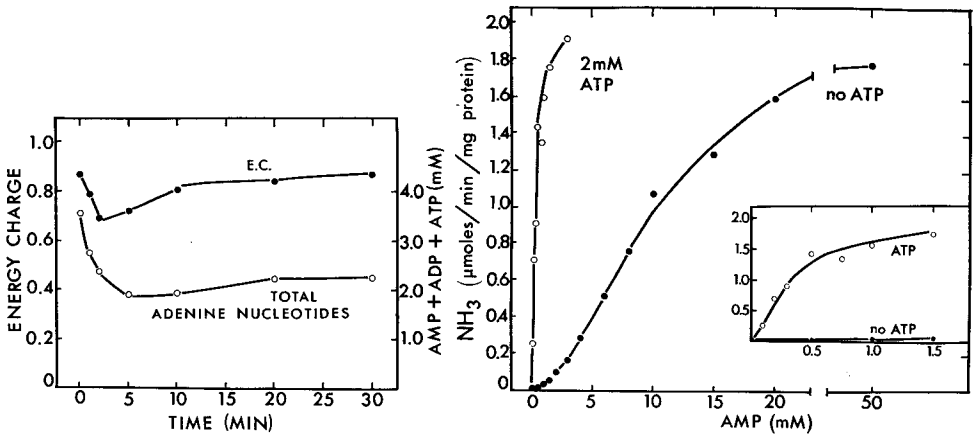

FIG. 1 (*left*). Changes in the adenine nucleotide concentration and energy charge (*E.C.*) value in liver following intravenous injecton at zero time of 1 mmole of D-fructose into female rats weighing 150 to 200 g. Calculated from data of Raivio *et al.* (7).

FIG. 2 (*right*). Rate of the reaction catalyzed by rat liver adenylate deaminase as a function of substrate concentration; effect of ATP. The *inset* shows the low substrate portion of the figure expanded. The reaction mixture contained 100 mM imidazole·Cl buffer, pH 6.8, 100 mM K^+, 4 mM Mg^{2+}, 1 mM citrate, 0.3 mM GTP, 2 mM ATP where indicated, and AMP as specified. The mixture also contained 4 mM α-ketoglutarate, 0.13 mM DPNH, and 0.5 mg (50 μl) of commercial glutamate dehydrogenase (Boehringer enzyme in 50% glycerol) required for the coupling reaction. After incubation of the assay mixture with glutamate dehydrogenase for 10 min at 37°, the reaction was started by adding 6.6 μg of partially purified AMP deaminase. Ammonia generated in the AMP deaminase reaction was measured by coupling it to the glutamate dehydrogenase reaction and following DPNH oxidation at 340 nm using a Varian model 135 spectrophotometer with recorder. Glutamate dehydrogenase has a low affinity for NH_4^+, and there is an initial period (2 to 3 min) of increasing velocity before linear rates are established. The duration of the initial period is inversely proportional to the amount of coupling enzyme used, but the subsequent linear rate is constant over a wide range of concentrations of glutamate dehydrogenase. In the presence of GTP, which inhibits glutamate dehydrogenase, the linear region was unchanged at the enzyme concentrations used, but a longer period was required before it was attained.

Liver AMP deaminase was purified approximately 50-fold using a modification of the method of Smith and Kizer (9). The gel filtration step in their procedure was omitted, and a 17-hour incubation at 37° was substituted for their 5-min incubation at 55°. The purified enzyme contained negligible amounts of adenylate kinase, ATPase, adenosine deaminase, and 5′-nucleotidase activities. The enzyme was stored at 0° in 0.2 M Tris-Cl and 10 mM ATP at pH 7.4 without appreciable loss of activity for 2 months. Prior to use the enzyme was dialyzed against 20 mM Tris-Cl, pH 7.4, and 0.1% β-mercaptoethanol to remove ATP.

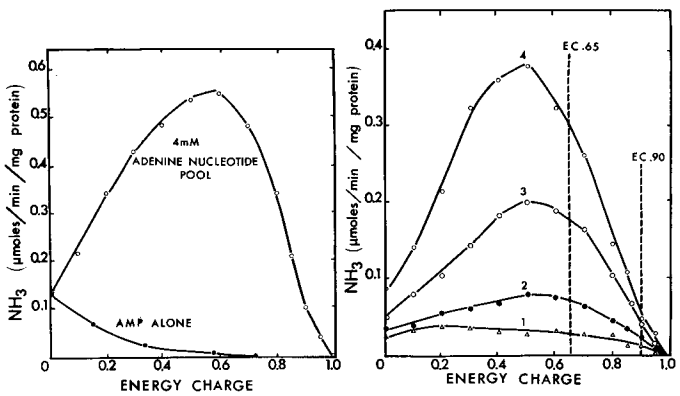

FIG. 3 (*left*). Response of rat liver AMP deaminase to variation in the adenylate energy charge at a total adenine nucleotide concentration of 4 mM. The *upper curve* shows the energy charge response of the enzyme with a 4 mM adenylate pool, and the *lower curve* shows the rate of the reaction at concentrations of AMP alone corresponding to the AMP concentration in a 4 mM adenylate pool at energy charge values indicated in the figure. The relative amounts of AMP, ADP, and ATP at different energy charge values were calculated on the basis of a value of 0.8 for the equilibrium constant of the adenylate kinase reaction. The assay mixture contained 100 mM imidazole·Cl, pH 6.8, 100 mM K^+, 8 mM Mg^{2+}, 4 mM adenine nucleotides (*upper curve*) or 0 to 4 mM AMP (*lower curve*), 25 μg of partially purified liver AMP deaminase, and the coupling system described in the legend to Fig. 2.

FIG. 4 (*right*). Response of rat liver adenylate deaminase to variation in the energy charge (*EC*) at different adenine nucleotide pool sizes in the presence of other effectors at presumed physiological concentrations. The reaction mixture contained 100 mM imidazole·Cl buffer, pH 6.8, 100 mM K^+, 0.5 mM free Mg^{2+}, 0.4 mM citrate, 0.3 mM GTP, 0.2 mM IMP, 5 mM P_i, 25 μg of partially purified liver AMP deaminase, adenine nucleotides at a total concentration of 4, 3, 2, or 1 mM as indicated by figures identifying the curves and at energy charge values indicated on the *abscissa*, and the glutamate dehydrogenase coupling system described in the legend to Fig. 2.

observed in intact liver. The *left-hand* part of the figure is probably of no physiological significance, since it is very unlikely that energy charge values below 0.6 ever occur in liver *in vivo*.

The effects of a number of known or possible modifiers of adenylate deaminase were tested under conditions similar to those in Fig. 3. The total adenine nucleotide concentration was held constant at 3.2 mM, and the energy charge value was varied between 0.6 and 0.95. Orthophosphate (1 to 6 mM) and K^+ (50 to 150 mM) inhibited the reaction 20 to 65% under these conditions; GTP (0.1 to 0.5 mM), IMP (0.2 to 1 mM), and citrate (1 to 10 mM) inhibited 20 to 30%; and lactate (1 to 5 mM) had no effect. There also seemed to be a trend toward greater inhibition at higher energy charge values.

Since the MgATP complex is the activating species for adenylate deaminase, the energy charge response is a function of the Mg^{2+} concentration when it is lower than that of ATP. The main result of reducing the Mg^{2+} concentration in the assay is severe inhibition of the reaction at high values of energy charge (above 0.8). The concentration of free Mg^{2+} (not complexed with proteins, adenylates, or other chelators) in liver is estimated to be 0.5 to 1 mM (13). At this level of Mg^{2+}, even a relatively large change in the magnesium ion concentration causes only a minor change in the energy charge response of adenylate deaminase.

The family of curves in Fig. 4 shows the response of the enzyme to variation in energy charge at different adenylate pool sizes in the presence of concentrations of other effectors assumed to be in the physiological range: 5 mM P_i, 100 mM K^+, 0.4 mM citrate, 0.3 mM GTP, 0.2 mM IMP (8), and 0.5 mM free Mg^{2+}. In estimating the total Mg^{2+} concentration required to lead to

a free Mg^{2+} concentration of 0.5 mM, the amount of Mg^{2+} binding to ATP^{4-}, $HATP^{3-}$, ADP, AMP, citrate, GTP, IMP, and α-ketoglutarate was calculated, based on dissociation constants used by Veloso *et al.* (13), at an energy charge value of 0.9 for each of the four different adenylate pool sizes. For simplicity, and also since it is not known how well the free Mg^{2+} concentration is stabilized *in vivo*, the total Mg^{2+} concentration was held at a fixed level for each of the four curves. Therefore the free Mg^{2+} concentration increases for each of the curves as the energy charge value (and the ATP concentration) decreases. But since the adenylate deaminase energy charge response is insensitive to a change in Mg^{2+} concentration at this level, the results are not significantly affected.

It is obviously metabolically desirable that the cell be protected against complete depletion of the adenine nucleotide pool. Such protection is observed experimentally. After injection of fructose, Raivio *et al.* (7) found that depletion of the adenylates ceased when the pool size reached 1.9 mM, and Woods *et al.* (8) found in perfused liver a terminal pool size of 1.2 mM. The lowest energy charge values attained in the two studies were 0.69 and 0.61, respectively. It seems likely that the response of adenylate deaminase to the concentration of ATP is an evolutionary adaptation that safeguards against excessive depletion of the pool of adenine nucleotides. A sigmoid response to the concentration of the substrate, AMP, would seem to be sufficient to protect against sharp falls in the value of energy charge, but such a response might lead to near-total depletion of the pool under conditions of energy stress.

Dependence of the rate of deamination on the concentration of ATP is essentially dependence on the pool size; this response causes the rate of depletion of the pool to decrease sharply well short of total depletion. The curves of Fig. 4 demonstrate that at an energy charge value of 0.65 the rate of the reaction decreases by a factor of 12 in going from an adenylate pool of 4 to 1 mM. Interpolation in the figure suggests that at an energy charge of 0.65 and pool size of 1.5 mM (approximately the conditions when depletion of the adenylate pool ceases in the livers of fructose-injected rats or in fructose-perfused liver) the rate of the deaminase reaction is about equal to that observed under the approximately physiological conditions of 0.9 energy charge and a total pool of 4 mM, although the AMP concentration is about 3-fold higher in the former case (0.27 mM compared to 0.09 mM). Thus we suggest that liver adenylate deaminase functions as a sophisticated self-limited safety device. Protection of the cell against drastic short term decrease in energy charge is at the expense of the adenine nucleotide pool, but the dependence of the rate of deamination of AMP on the concentration of ATP (the major constituent of the pool) provides a built-in limit on how far the pool size will be reduced.

Our results correlate well with metabolic adenine nucleotide changes observed *in vivo*, and thus are consistent with the role that we propose for adenylate deaminase. The decrease in energy charge reported *in vivo* stops at a value where the adenylate deaminase reaction was found to proceed *in vitro* at close to its maximal rate, and the depletion of the adenylate pool ceases at a level (1 to 2 mM) where the rate measured *in vitro* at an energy charge of 0.65 is approximately the same as under normal physiological conditions. The observed rate of 0.3 μmole of AMP converted per min per mg of protein at 37° (Fig. 4) may be calculated to correspond to a rate *in vivo* of approximately 0.2 μmole per min per g wet weight by assuming a 50-fold purification, a 30% recovery of enzyme activity, protein equal to 50% of total dry weight, and dry weight 20% of wet weight. At this rate, it would take about 15 min to remove 1.5 μmoles of AMP from the adenylate pool. This compares reasonably well, considering the approximations involved in extrapolation from enzyme experiments to the situation *in vivo*, with the 5 min required for the removal of 1.5 μmoles of AMP in the experiment of Raivio *et al.* (7) shown in Fig. 1.

It is not known whether the adenylate deaminase reaction proceeds at a significant rate under normal physiological conditions, thus participating in a futile cycle (14) of relatively low velocity as would be suggested by our results (Figs. 3 and 4). Controls and interactions not operative in our system *in vitro* may cause the reaction to be effectively totally inhibited *in vivo* except under conditions of metabolic stress.

The adenylate pool has been shown in several tissues and organisms to decrease when the energy charge drops (4–8). Thus it appears that stabilization of the energy charge at the expense of the concentration of the adenylate pool may be a general phenomenon. Ascites tumor cells are known to produce IMP following addition of glucose or of deoxyglucose (15), and appear to have a system similar to that of liver. The properties of adenylate deaminase from ascites cells are very similar to those of the liver enzyme.[2] In heart, however, removal of AMP may be accomplished by the AMP 5′-nucleotidase reaction (16, 17). The product of this reaction, adenosine, has been suggested to be a potent vasodilator, which would help further to restore high energy conditions.

The inhibition of adenylate deaminase by inorganic phosphate may also be of physiological significance. Coincident with the drop in energy charge the P_i concentration was observed to undergo a transient 65% decrease following fructose injection (7). This would release the inhibition of the enzyme by phosphate and increase further the rate of deamination. The generality of this reinforcement of the energy charge by variation in phosphate concentration seems open to question, however. Under many conditions, including starvation, it seems likely that a decrease in energy charge may be accompanied by an increase in the concentration of orthophosphate.

Because of its role in the regulation of blood carbohydrate, liver may be faced with recurring minor energy charge crises when the concentration of a phosphoryl acceptor in the blood rises abruptly. The properties of liver adenylate deaminase that we describe may have evolved as part of the system that protects against wide excursions of energy charge under those conditions. Because of the limited amount of adenine nucleotides present in the cell, stabilization of charge at the expense of pool size cannot be a long term effect; it can protect only against sharp drops in charge during the transient period while a new steady state, in which use and regeneration of ATP are again equal, is established. Many other enzymes, including those that participate in the synthesis *de novo* of adenylates, must of course also be involved in these changes and in the ultimate recovery of the pool size.

Vertebrates are adapted to ingestion of food at intervals rather than continuously. Thus starvation in vertebrates develops slowly and cannot be an immediate crisis leading to the danger of a sharp transient drop in energy charge. In microorganisms, on the other hand, and especially in bacteria, the onset of starvation can be, and probably frequently is, immediate. Thus in bacteria, starvation may be expected to lead to a transient energy crisis like that caused when liver is flooded by a phosphoryl acceptor. We earlier found that the energy

[2] A. L. Miller, unpublished observation.

charge in *Escherichia coli* drops relatively little on starvation while the adenine nucleotide pool decreases to about 50% of its initial size (4). This finding is consistent with protection of charge by removal of AMP. However, *E. coli* and many other bacteria seem to lack adenylate deaminase; thus stabilization of charge at the expense of pool cannot be effected in these organisms by the mechanism proposed for liver. A likely answer to this dilemma is suggested by the extremely interesting finding by Schramm and Leung (18) that adenylate nucleosidase (which cleaves AMP to adenine and ribose 5-phosphate) occurs in *Azotobacter*, *E. coli*, and other bacteria, and responds to variation in adenylate energy charge and in ATP level in very much the same way as does adenylate deaminase from liver. Thus it seems a reasonable working hypothesis at present that removal of AMP as a protection against sharp drops in energy charge may be a general phenomenon, but that the reaction by which the removal is effected varies among organisms and very likely also among tissues.

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