RAPID CHANGES IN INITIATION-LIMITED RATES OF PROTEIN SYNTHESIS IN RAT THYMIC LYMPHOCYTES CORRELATE WITH ENERGY CHARGE

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SUMMARY. Depriving rat thymocytes of energy-providing substrates for 2 hr results in a 75-80% drop in rates of protein synthesis and a shift of ribosomes from active polysomes to inactive monomers and dimers. Glucose prevents these changes or, when added to starved cells, rapidly reverses them. Restoration of protein synthesis is associated with reversal of the 7% decline in the adenylate energy charge seen in starved cells. The data is consistent with the hypothesis that glucose increases initiation in starved cells, probably via effects on the balance of adenine nucleotides. Data with other substrates support this concept. The inability of glucose to fully restore energy charge in the presence of glucocorticoids or rotenone correlates with the limitation of protein synthesis.

Our previous studies have shown that a number of circumstances that produce small changes in levels of adenine nucleotides lead to rapid changes in the overall rate of protein synthesis (1-3). Regulation of protein synthesis has been proposed by Atkinson to be linked to the adenylate energy charge $[(ATP) + \frac{1}{2}(ADP)]/[(ATP) + (ADP) + (AMP)]$ (cf 4). Here we further investigate these relationships in normal mammalian cells under a variety of nutrient and energy-limited conditions. We show that appropriate changes in the adenylate energy charge precede or accompany rapid alterations in protein synthesis. These changes are accompanied by shifts in the polyribosomal profiles consistent with the hypothesis that small changes in the balance of adenine nucleotides exert a physiologically relevant influence on rates on initiation. Accompanying changes in rates of elongation may also occur.

MATERIALS AND METHODS

Suspensions of isolated thymus cells were prepared gently and rapidly and incubated in Krebs-Ringer bicarbonate buffer as previously described (1-3). Incorporation of 2,3[^{3}H]valine (0.1 mg/ml, 5.0 μCi/ml) into whole cell protein was determined as reported (1-3). We have presented evidence that with these conditions incorporation of radioactivity reflects rates of protein synthesis,

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not changes in amino acid pools (1,2,5).[1] Chemicals (reagent grade) were purchased from standard suppliers; enzymes and cofactors from Boehringer-Mannheim; and 2,3[^{3}H]valine and 4,5[^{3}H]leucine from Amersham-Searle.

Preparation of Polysomes. Cells were lysed by addition of 4 volumes of cold solution A (5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM EDTA, pH 7.0). The [Mg^{++}] of the post-nuclear supernatant was increased to 50 mM. After 30 min at 0°, polysomes were pelleted by centrifugation at 15,000 x g for 10 min, rinsed, and resuspended in TKM buffer (20 mM Tris-Cl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$) with 1 mM dithiothreitol and 0.1 mM EDTA. The resuspension was clarified by a 5-min centrifugation at 2000 x g. All solutions were pretreated with 0.01% diethylpyrocarbonate to inactivate RNase.

Sucrose Gradient Centrifugation. For the analysis of polysomal profiles, equal amounts (A_{260}) of polysomal preparations were layered onto 15-50% convex sucrose gradients (4 ml 15% sucrose-TKM: 8 ml 50% sucrose-TKM). The gradients were centrifuged for 45 min at 41,000 RPM in a SW41 Ti rotor. Absorbance at 254 nm was recorded using an ISCO gradient fractionator and absorbance monitor. Areas of the scans were measured using both planimetry and weighing cutouts of the areas; baseline was determined from blank gradients.

Assays for ATP, ADP, and AMP. Triplicate or quadruplicate 1 ml aliquots of cell suspension were precipitated with cold perchloric acid and adenine nucleotides were assayed by standard enzymatic methods (6) with minor modifications and special attention to careful neutralization of each sample to pH 7.6 ± 0.1 with a microtitrator.

RESULTS

Earlier studies have demonstrated that isolated rat thymic lymphocytes incubated with glucose synthesize protein at a nearly linear rate for at least two hours; whereas, without glucose, protein synthesis declines by 75-80%. Addition of glucose to such starved cells restores protein synthesis within min to a rate approaching that seen in fresh cells (1-3).

A polysomal profile typical of that seen in freshly prepared thymocytes is shown in Fig. 1A: the polysomes comprise about 40% of the total ribosomal material, and the 40S plus the 60S subunits about 10%, with the remainder divided between large 80S and dimer peaks. Incubation with glucose for 2 hr, which maintains the overall rate of protein synthesis (see above), also maintains the polysomal distribution (Fig. 1B). Here, radiolabeled amino acids are primarily incorporated into nascent peptide chains associated with large polysomes; little labeling of the 80S and dimer regions is observed. Incubation of cells without glucose for 2 hr results in changes in the polysomal profile consistent with the lower rates of protein synthesis seen. Incorporation of

[1]Similar conclusions have been reached from cell-free protein synthesis experiments (W.A. Guyette and D.A. Young, to be published separately).

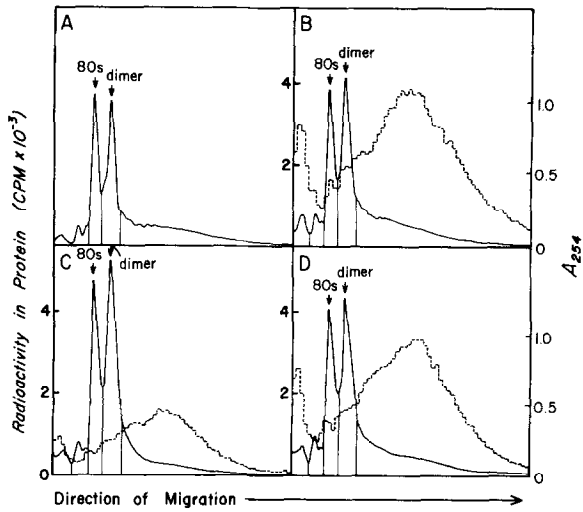

FIGURE 1. Sucrose gradient sedimentation profiles of polysomes from: A) freshly prepared cells incubated for only 10 min; B) cells incubated with glucose for 125 min; C) cells incubated for 125 min without substrate; and D) cells incubated for 115 min without substrate, then given glucose for 10 min. Nascent peptides were labelled by addition of [^{3}H]leucine (0.5 µCi/ml; 50 Ci/mmol) at 120 min (*broken lines*).

amino acids into nascent chains is decreased as are the size and the number of polysomes (Fig. 1C). The restoration of protein synthesis in starved cells given glucose for 10 min is accompanied by reestablishment of the original polysomal distribution (Fig. 1D).

In order to determine more precisely the relationships between changes in the balance of adenine nucleotides and associated alterations in protein synthesis, we have examined these parameters concurrently during starvation and restoration conditions in conjunction with changes in polysomal profiles. The changes in the polysomes resulting from deprivation of glucose are quantitated in Fig. 2A and the reversal of these changes by glucose in Fig. 2B. Within 2.5 min after glucose there is a significant movement of ribosomes from the 80S and dimer regions into the polysomes. This shift continues through 30 min by which time the proportion of ribosomal material in polysomes nearly equals that seen in cells provided with glucose from the start.

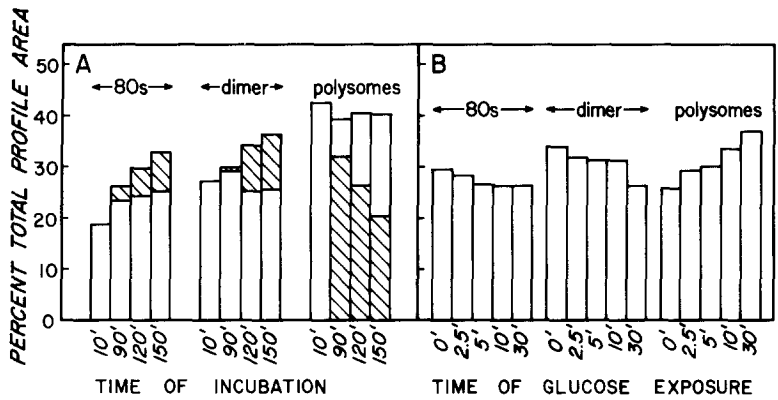

FIGURE 2. Relative changes in the 80S, dimer, and polysomal regions of sucrose gradient profiles isolated from cells during substrate deprivation and glucose-induced restoration. The areas of the 80S, dimer, and polysome regions are expressed as percent of the total area of the profile using divisions like those depicted in Figure 1. A) Cells were incubated for the indicated times either with glucose (*clear bars*) or without (*hatched bars*); B) cells were incubated for 120 min without substrate and then given glucose for the times indicated.

The rates of protein synthesis and the adenylate energy charge in this same experiment are shown in Fig. 3 (*circles*). Beginning with the bottommost symbol, the *circles* represent: (No G) cells incubated without glucose for 150 min; (No G) without glucose for 120 min; (G 2.5') with glucose for 2.5 min after 120 min without; (G 30') glucose for 30 min after 120 min without; (G 150') glucose for entire 150 min; (G 120') glucose for entire 120 min. The 80% drop in rates of protein synthesis in cells incubated without glucose for 2 hr is accompanied by a 6.5% drop in energy charge (0.932 to 0.871), due to a 27% drop in ATP, a 39% increase in ADP, and a 175% increase in AMP. Glucose reverses these changes; by 2.5 min the energy charge has risen to 0.922 and rates of protein synthesis have already more than doubled. The steep portion of the curve in Fig. 3 was drawn to fit these points.

This apparent association of large changes in protein synthetic rates with subtle changes in energy charge was tested by varying energy charge and protein synthesis in various ways, and the results are plotted in this same figure. Glucose-fed cells exposed to a physiologically relevant glucocorticoid activity

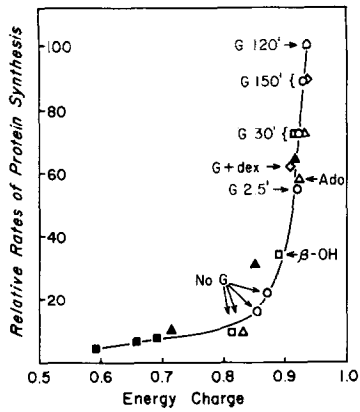

FIGURE 3. Rates of protein synthesis versus energy charge. Each of the four types of symbols represent data from one experiment. Experiment 1 (O): glucose for 120 min and 150 min no glucose for 120 min and 150 min, or glucose for 2.5 min and 30 min after 120 min without. Rates of protein synthesis are expressed as percent of rates in cells incubated with glucose (5.5 mM) for 2 hrs. Since there is variation in the absolute rates of protein synthesis between experiments, the data from Experiments 2-4 were normalized to Experiment 1 by assigning equal relative rates of protein synthesis to the plus glucose conditions. Experiment 2 (◇): glucose or glucose + dexamethasone (10^{-7}M) for 150 min. Experiment 3 (□ , ■): β-hydroxybutyrate (5.5 mM), glucose, or glucose + rotenone (filled symbols; 350 nM, 535 nM, or 720 nM) added at 120 min. Incubation ended at 150 min. Experiment 4 (△ , ▲) same protocol as Experiment 3 except adenosine (2.5 mM) replaced β-hydroxybutyrate, and rotenone levels were 70 nM, 140 nM, or 280 nM.

(dexamethasone 10^{-7}M) for 2.5 hr exhibit a rate of protein synthesis 30% lower than the control and an energy charge of 0.908 versus 0.928 (compare *diamonds*, G, vs. G + dex). An important role of glycolytic ATP is suggested by experiments in which provision of β-hydroxybutyrate to starved cells restores neither protein synthesis nor energy charge as effectively as glucose (compare *squares*, β-OH vs. G 30'). Similar results have been seen with pyruvate and lactate (1,2).

Adenosine, like glucose, provides glycolytic ATP along with oxidative ATP (7) With adenosine, levels of ATP are actually higher than with glucose (here by 16%). Despite the higher levels of ATP, adenosine is slightly less effective at restoring energy charge, and this is reflected in the slightly lesser restoration of protein synthesis (compare *triangles*, Ado vs. G 30').

Another approach was used to examine the influence of adenine nucleotides

on rates of protein synthesis. The ability of glucose to restore energy charge was limited (partially or totally) by partially blocking mitochondrial ATP production by the simultaneous addition of low doses of rotenone. In these experiments even the slightest inhibition of the restoration of energy charge is associated with a reduction in the restoration of protein synthesis by glucose (compare *filled triangles* and *squares* with the *open symbols*).

DISCUSSION

We have attempted to establish a connection between the adenylate energy charge and protein synthesis in normal, intact mammalian cells. Fig. 3 demonstrates how tightly energy charge is controlled within the cell and how subtle changes within the physiological range of energy charge (0.75 - 0.95) (8) correspond to large changes in rates of protein synthesis. In our hands energy charge is a better index of the effects of adenine nucleotides on protein synthesis than levels of ATP alone or ratios of individual nucleotides. Our findings further suggest that both glycolytic and oxidative ATP are required for the maintenance of maximal rates of protein synthesis and support earlier proposals that glucocorticoid hormones influence rates of protein synthesis through an inhibition of ATP production (3). It is interesting that the relation between energy charge and rates of protein synthesis depicted in Fig. 3 closely resembles that seen previously in *E. coli* (4), suggesting that a close association between protein synthesis and the balance of adenine nucleotides may be universal in biology.

Nutrients are known to affect rates of protein synthesis in a variety of mammalian tissues. In some cases these changes can be attributed to altered rates of initiation (9-11). As rates of protein synthesis decrease in the absence of glucose in thymus cells, so does the proportion of ribosomes associated with polysomes and the average polysome size. This pattern could occur if elongation rates were increased, which is not compatible with decreased rates of synthesis. More likely, rates of initiation are decreased.[1] During restoration, the 3- to 4-fold increase in the rate of protein synthesis cannot be

completely accounted for by the shift of ribosomes to the polysomal region, therefore an increase in elongation rates probably accompanies the increase in initiation. The polysomal distributions presented here indicating that a large fraction of ribosomal material is not associated with large, actively synthesizing polysomes is apparently typical of lymphoid tissues[2] (12-14). This suggests that either initiation is rate-limiting and/or that there is a large reservoir of ribosomes incapable of participating in protein synthesis.

The primary energy source for protein synthesis is GTP (15), which is involved in several processes in the initiation sequence that are inhibited by GDP. Thus, the rate of initiation may be controlled by the GTP:GDP ratio, which in turn may be regulated by the adenine nucleotide ratios via enzymatic linkage between guanylate pools and the larger adenylate pools. Walton and Gill have presented evidence that formation of ternary complex (Met-tRNA$_f$·GTP· initiation factor 2) and the subsequent binding of mRNA in a cell-free system are dependent on GTP:GDP ratios (16). Our data, which link rapid changes in initiation to changes in the energy charge suggest that such a mechanism may be a physiologically important regulatory mechanism in intact mammalian cells. Adenine nucleotides may also regulate rates of protein synthesis by altering initiation complex formation directly (17), amino acylation of tRNA, or the availability of mRNA or blocked ribosomal subunits.

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[2]The distribution of nascent chains indicates that this is not an artifact of degradation during preparation. This conclusion is supported by experiments that show very similar polysomal distributions in direct homogenates from whole thymuses quick frozen in liquid nitrogen and prepared in buffers of various ionic compositions or in the presence of heparin to inhibit RNase.

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