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THE EXTENT OF PHOSPHORYLATION OF THE ACIDIC 60S RIBOSOMAL PHOSPHOPROTEIN, Lγ, IN KREBS II ASCITES CELLS AND IN THE SKELETAL MUSCLE OF NORMAL AND DIABETIC RATS

David P. Leader, Amanda A. Coia and Laila H. Fahmy

Department of Biochemistry, University of Glasgow,
Glasgow G12 8QQ, Scotland, U.K.

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SUMMARY

The phosphorylated and non-phosphorylated forms of the 'acidic' 60S ribosomal phosphoprotein, Lγ, have been resolved using the recently devised method of two-dimensional "sweep" gel electrophoresis. This has enabled us to demonstrate that the previously reported decrease in the labelling of this protein with (^{32}P)orthophosphate in ascites cells incubated in a medium lacking glucose reflects a real alteration in the extent of phosphorylation, rather than a change in the turnover of the phosphate. The method has also allowed comparison of the phosphorylation of Lγ in unlabelled ribosomes from the skeletal muscle of normal and diabetic rats, but here no alteration in the extent of phosphorylation was apparent.

INTRODUCTION

Although the existence of a phosphoprotein on the 40S subunit of eukaryotic ribosomes is well established (1 - 5), there has been considerable uncertainty whether the (^{32}P)-labelled band observed on one-dimensional gels of protein from the 60S subunit (1,6,7) represented a true ribosomal phosphoprotein. This was because the ^{32}P did not appear to migrate with any of the standard ribosomal proteins which are displayed by two-dimensional gel electrophoresis. Recently, however, several groups have observed a 60S phosphoprotein on two-dimensional gels designed to detect acidic ribosomal proteins (8 - 10), and this has caused considerable interest, as the protein (which we call Lγ) appears to be the eukaryotic equivalent of the functionally important E. coli ribosomal proteins, L7 and

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L12 (11,12). It is not difficult to envisage a role for the phosphorylation in modulating the activity of the protein, but there is as yet no experimental basis for this idea, and indeed the extent of phosphorylation of Lγ does not generally appear to show much variation (13). The one major exception to this that we have observed is in Krebs II ascites cells deprived of glucose, where Lγ, analysed on one-dimensional gels, showed a marked decrease in labelling by (^{32}P)orthophosphate (14). We felt that it was necessary to establish whether this change in labelling represented a real alteration in the extent of phosphorylation of Lγ; for, if this proved to be so, it would be interesting to determine whether a similar dephosphorylation might explain the functional alteration observed in the 60S subunit of ribosomes isolated from the skeletal muscle of rats with experimental diabetes (15). We describe here studies with a system of gel electrophoresis (13) which we show can resolve the phosphorylated and non-phosphorylated forms of Lγ, and has thus enabled us to answer these questions.

METHODS

Most of the methods used in this work have been described elsewhere. These include: the maintenance of the Krebs II ascites cells (5), their incubation and labelling with (^{32}P)orthophosphate (14), and the isolation of their ribosomes (16); the induction of diabetes in rats by injection of streptozotocin (17), the isolation of ribosomes from rat skeletal muscle (18), and the analysis of these on sucrose density gradients (17); the dissociation of ribosomes into subunits and the extraction of ribosomal proteins (16), and the analysis of the proteins by two-dimensional 'sweep' gel electrophoresis (13).

Dephosphorylation of ascites Lγ by alkaline phosphomonoesterase (EC 3.1.3.1) was as follows. A portion of ribosomes (35 mg), isolated from ascites cells incubated for 3 hr in medium lacking glucose and amino acids, was suspended in 10 ml buffer containing Tris-HCl, pH 8 (10mM), KCl (80mM), $MgCl_2$ (10mM) and incubated with 5 mg alkaline phosphomonoesterase (Grade I from calf-intestine - Boehringer Corporation Ltd.) for 120 min. The ribosomes were then sedimented for 120 min at 165,000 g in a Beckman Ti50 rotor and their subunits dissociated and protein extracted as indicated above.

RESULTS AND DISCUSSION

In our previous studies we found that Lγ was not labelled by (^{32}P)or-

thophosphate in ascites cells incubated in medium lacking glucose (14). However, as we only performed one-dimensional gel analysis of the protein, the results might have been due to an alteration of the rate of turnover of the phosphate rather than a change in the extent of phosphorylation. This point was of some importance as we are not aware of any other circumstances where the phosphorylation of Lγ is completely inhibited. We therefore decided to examine Lγ by the method of "sweep" gel electrophoresis we have recently devised (13). The essential feature of this method is the concentration of acidic proteins into a narrow band in the first dimension. Basic proteins (which provide useful reference points) also migrate into the first dimension gel and are resolved mainly according to size in the 18% acrylamide of the second dimension. However it appeared likely that the phosphorylated and non-phosphorylated forms of Lγ would be resolved on the basis of charge in the second dimension, because of the proximity of the isoelectric point of this acidic protein to the low pH of the gel (pH 4.5).

The results of the analysis of ascites Lγ are shown in Fig. 1. In ribosomal protein from the cells incubated without glucose, Lγ migrated as a single stained spot (Fig. 1(ii)) and no radioactivity was detected in the position corresponding to this on the autoradiograph, although a very faint radioactive spot was visible just above (Fig. 1(i)). The labelling of Lγ is only prevented if amino acids are present in the medium and, because of the extensive production of lactic acid if glucose is added, it is more convenient to label Lγ in a medium lacking both glucose and amino acids (14). When ribosomal protein from cells labelled in such a medium was subjected to two-dimensional analysis (Fig. 1(iii) and (iv)), there were two stained spots, only the upper one of which was labelled. Comparison of the position of the stained Lγ spots in Figs. 1(ii) and (iv), using the basic proteins as reference points, indicates that the single stained spot in the protein from cells deprived of glucose alone corresponds to the lower of the

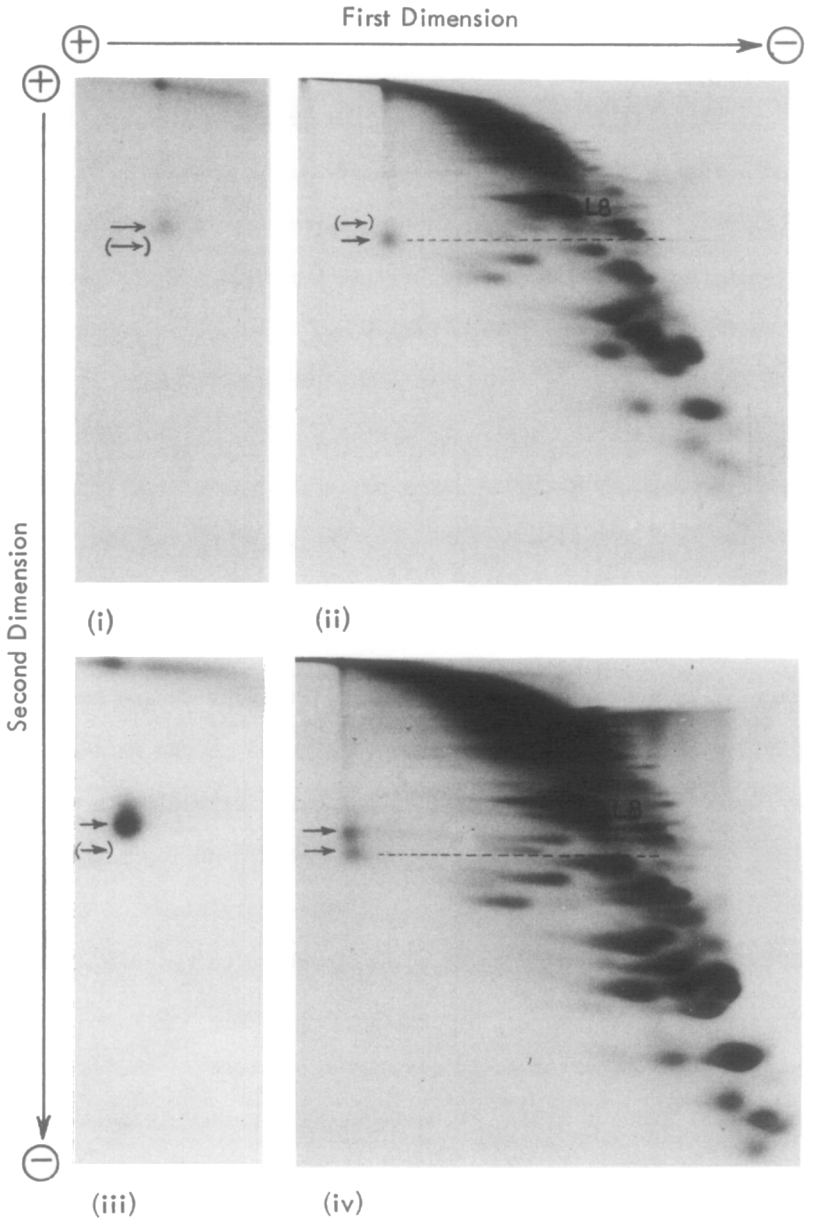

Figure 1. Two-dimensional "sweep" gel electrophoresis of protein extracted from the 60S subunits of ascites cells incubated with (^{32}P)orthophosphate. The ribosomal protein (200 µg) analysed had been extracted from: (i) Cells incubated in Earle's saline, lacking glucose but supplemented with amino acids - section of autoradiograph corresponding to left-hand side of (ii) stained gel; (iii) Cells incubated in Earle's saline without glucose or amino acids - section of autoradiograph corresponding to left-hand side of (iv) stained gel. The arrows are at identical positions in each pair of frames, a parenthesis indicating that no spot was visible. The broken lines and protein L8 are marked to allow comparison between (ii) and (iv), and these and Fig. 2 (ii).

two spots in the protein from the cells deprived of both glucose and amino acids. The simplest interpretation of these results is that the upper labelled spot in Fig. 1(iii) and (iv) is the phosphorylated derivative of the unlabelled lower one; and that in ascites cells incubated in medium lacking only glucose, Lγ is indeed in the unphosphorylated state.

This interpretation of the electrophoretic behaviour of the proteins is supported by other evidence. Fig. 2(i) shows that acidic proteins differing by a single charge (E. coli ribosomal proteins L7 and L12 (19)) are resolved in this gel electrophoretic system. Fig. 2(ii) shows that when ribosomes from ascites cells in which Lγ is phosphorylated (cf Fig. 1(iv)) are treated with alkaline phosphatase, the upper of the two stained Lγ spots - the putative phosphorylated form - disappears.

In our original report we presented detailed evidence that the dephosphorylation of Lγ (and the de novo phosphorylation of S3 and L14 which accompany this) is specifically due to lack of glucose in the medium in which the ascites cells are incubated (14). Another situation in which cells are deprived of glucose is in the skeletal muscle during diabetes, and we wished to know whether there was also dephosphorylation of Lγ in these circumstances. This was of special interest to us because of the translational control of protein synthesis operating here (20), and the fact that this has been reported to involve an alteration in the activity of the 60S ribosomal subunit (15). However we had previously been unable to investigate the matter because it had not been possible to label the phosphoryl group of Lγ sufficiently by injecting (^{32}P)orthophosphate into rats. Now, using two-dimensional "sweep" gel analysis to resolve the phosphorylated and non-phosphorylated forms of Lγ, it was possible to examine this question using unlabelled ribosomal protein. The results of such an analysis are shown in Fig. 3, where it can be seen that Lγ migrates as two stained spots, of similar relative position and intensity in ribosomal protein from the skeletal muscle of both normal and streptozotocin-diabetic rats.

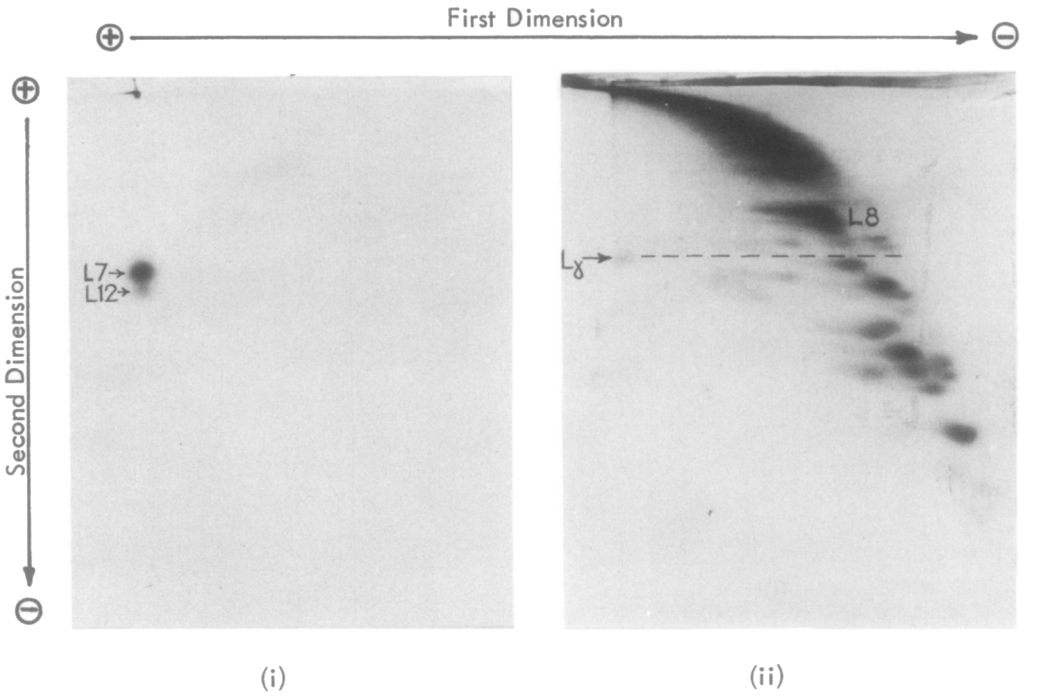

Figure 2. Effect of alteration in charge on the mobility of acidic ribosomal proteins subjected to two-dimensional "sweep" gel electrophoresis (i) E. coli ribosomal proteins L7 and L12, (5 µg) (ii) Protein (200 µg) from ascites ribosomes, dephosphorylated as described in Methods.

Thus it would appear that diabetes does not produce, in skeletal muscle ribosomes, a change in the extent of phosphorylation of Lγ, similar to that produced by glucose-deprivation of ascites cells. This may be because of incomplete glucose-deprivation in skeletal muscle, where the elevated blood glucose concentration might exert a mass action effect on transport; or it may be related to an accompanying reduction in amino acid transport in diabetes, given that amino acids are required to produce the decreased phosphorylation of ascites Lγ (14). It is still possible, however, that a change in the phosphorylation of some other 60S ribosomal protein (perhaps L14) takes place in the skeletal muscle of diabetic rats, but that such a change is not detectable on two-dimensional gels, perhaps because of the protein being very basic. Because a phosphorylation/dephosphorylation mechanism could account for the speed

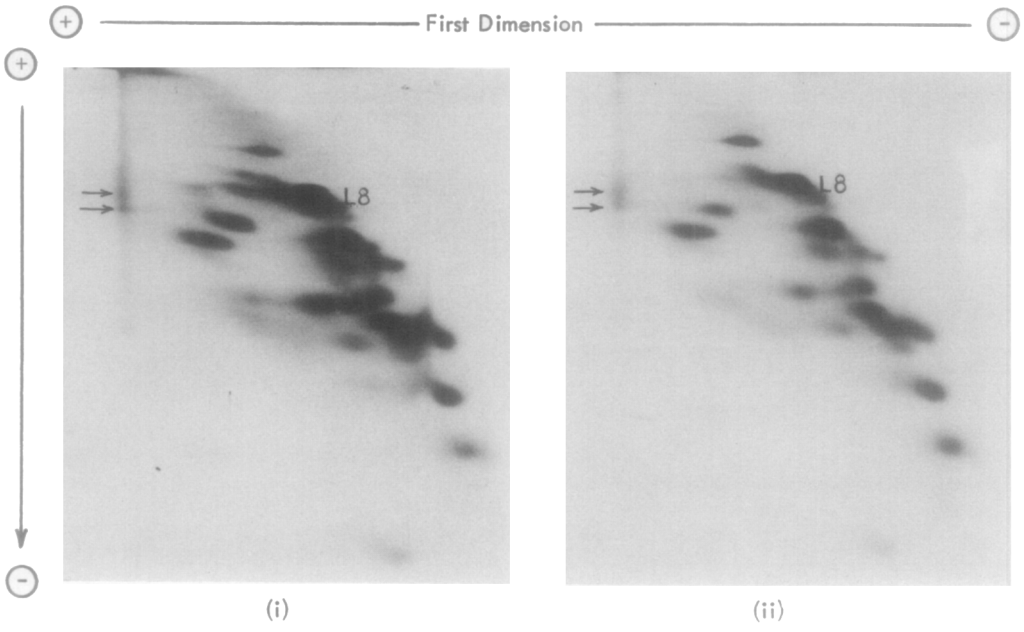

Figure 3. Two-dimensional "sweep" gel electrophoresis of protein extracted from the 60S subunits of skeletal muscle ribosomes. Ribosomal protein (200 µg) was from: (i) normal rats (ii) diabetic rats. The position of the Lγ spots are indicated by arrows, and L8 is marked as reference for comparison.

with which insulin is known to restore the activity of skeletal muscle ribosomes in diabetic rats (20), we are somewhat reluctant to discard this idea completely.

The demonstration in this report that the extent of phosphorylation of ribosomal protein Lγ does not differ in the skeletal muscle of normal and diabetic rats, although not establishing the nature of the lesion in protein synthesis in diabetes, does provide information relevant to an aspect of the phosphorylation of Lγ which has been subject to some dispute. This is the question of whether there is selective phosphorylation of this protein on monoribosomes, as Kabat reported for rabbit reticulocytes (1). As the proportion of polyribosomes differs markedly in the skeletal muscle of normal and diabetic rats (Fig. 4), our results would not support this idea, and in this respect they are consistent with direct examinations which we have performed on the phosphorylation of Lγ in the polyribosomes and monoribosomes of hamster fibroblasts (13).

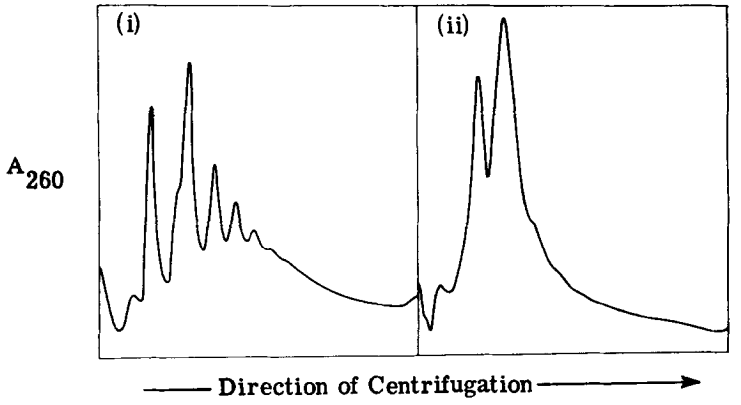

Figure 4. Analysis of polyribosomes from rat skeletal muscle. Ribosomes (150 µg) were resuspended in 0.2 ml buffer containing Tris-HCl, pH 7.6 (50mM), KCl (200mM) and $MgCl_2$ (5mM), applied to a 5.2 ml linear gradient of 15-45% (w/v) sucrose in the same buffer, and centrifuged at 4°C for 35 min at 234,000 g in a Beckman SW50.1 rotor. (i) ribosomes from normal rats (ii) ribosomes from diabetic rats.

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