Phosphorylation of branched-chain 2-oxo acid dehydrogenase complex in isolated adipocytes

Effects of 2-oxo acids

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Isolated adipocytes from rat epididymal fat-pads were incubated with [^{32} P] P_i , and intracellular phosphoproteins were then analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. A phosphorylated polypeptide of apparent M_r 46 000 was identified as the α -subunit of branched-chain 2-oxo acid dehydrogenase complex by immunoprecipitation using antiserum raised against the homogeneous E1 component of branched-chain 2-oxo acid dehydrogenase complex. Immunoprecipitation of this phosphoprotein is blocked in a competitive manner by purified branched-chain 2-oxo acid dehydrogenase complex. Peptide mapping of the isolated phosphoprotein indicates that two sites on the polypeptide are phosphorylated in the intact cells. Addition of branched-chain 2-oxo acids to the incubation medium causes diminution in the extent of labelling of both phosphorylation sites on the α -subunit, an effect presumably mediated via their known inhibitory action on branched-chain 2-oxo acid dehydrogenase kinase. These observations provide direct evidence for phosphorylation of branched-chain 2-oxo acid dehydrogenase complex in intact cells.

INTRODUCTION

Branched-chain 2-oxo acid dehydrogenase complex occupies a key position in amino acid metabolism, catalysing an irreversible step in the oxidation of the essential branched-chain amino acids [1,2]. During the past few years evidence has accumulated that the activity of this intramitochondrial complex is regulated by reversible phosphorylation of the α -subunit of the E1 component. The initial observations were made with rat heart mitochondria, when it was shown that depletion of ATP led to activation of the complex [3]. Subsequently several groups have studied regulation of the complex in isolated mitochondria from various tissues [4–8]. Furthermore, by using purified preparations of the complex it has been shown that the kinase which regulates the complex co-purifies with it [9–11] and that this kinase phosphorylates two sites on the α -subunit. Inactivation of the complex correlates with phosphorylation of one of these two sites [12,13] which are also phosphorylated within intact mitochondria [7].

To date, most of the work on acute regulation of the complex in cellular systems has been carried out by using activity measurements in perfused rat hearts [14–16]. With this system, the extent of phosphorylation of branched-chain 2-oxo acid dehydrogenase can be estimated by measurement of the activity of the complex in extracts prepared from these hearts before and after treatment of the extracts with preparations of a cytosolic protein phosphatase [15]. It has also been shown, however, that in perfused rat heart a polypeptide becomes phosphorylated which has mobility during SDS/polyacrylamide-gel electrophoresis identical with that of the α -subunit of branched-chain 2-oxo acid dehydrogenase complex purified from bovine kidney, and the authors of that work concluded that this phosphoprotein was indeed the α -subunit of the complex [16]. In a preliminary report [17] it was demonstrated that a protein

of M_r approx. 48 000, which becomes phosphorylated in isolated mitochondria, also is phosphorylated in isolated adipocytes. From effects of branched-chain 2-oxo acids on the extent of phosphorylation of that protein, it was concluded that the phosphoprotein was the α -subunit of the complex.

Isolated adipocytes represent a sensitive whole-cell system suitable for study of the acute regulation of the complex. We have developed a method for determination of the phosphorylation state of branched-chain 2-oxo acid dehydrogenase complex in these cells and show here that the α -subunit of the complex is phosphorylated at two sites and that the presence of physiological concentrations of 2-oxo acids in the incubation medium inhibits phosphorylation at both these sites.

MATERIALS AND METHODS

Adipocytes were prepared by a previously published method [18] from epididymal fat-pads taken from 200 g male Sprague–Dawley rats which had been starved overnight. Pads were digested with collagenase (1 mg/ml) for 60 min at 37 °C in Krebs–Ringer Hepes, pH 7.4 (as specified in [18]), containing 1.2 mM-phosphate, 2 mM-glucose, 400 nM-adenosine and 2% (w/v) defatted bovine serum albumin.

Adipocytes were isolated, washed and resuspended in the same buffer containing 50 μ M-phosphate and 1% defatted bovine serum albumin, and preincubated for 1 h in the presence of [^{32} P] P_i (100 μ Ci/ml) before addition of effector. Incubations (200 μ l, containing approx. 40 μ l of packed cells) in the presence or absence of effectors were terminated by spinning the cells (10 s at 1000 g) through a layer of dinonyl phthalate oil and removing the medium by aspiration. The cells were resuspended in 80 μ l of ice-cold 20 mM-sodium pyrophosphate, pH 7.0, 1% (v/v) Triton X-100, 1% (w/v) SDS, 2% (w/v) sodium deoxycholate, 1 mM-EDTA, 1 mM-EGTA, 5 mM-NaF,

20 μg of leupeptin/ml, 20 μg of pepstatin/ml, 10 μM -tosyl-lysylchloromethane ('TLCK'), 10 μM -tosylphenylalanylchloromethane ('TPCK'), 0.1 mM-phenylmethanesulphonyl fluoride and 1 mM-dithiothreitol and lysed by vortex-mixing for 15 s. An extract was obtained by centrifugation at 1000 g for 20 s to remove the oil and at 10000 g for 30 s to remove lipid. Samples (2.0×10^6 d.p.m.) of the infranatant (extract) were analysed by SDS/polyacrylamide-gel electrophoresis in a Tris/glycine buffer system [19] with 10% acrylamide in the running gel. After staining of protein with Coomassie Brilliant Blue R, the gels were dried and autoradiographed for 1–3 days by using Kodak direct-exposure X-ray film. Autoradiographs were subjected to densitometric scanning with a Bio-Rad densitometer (model 1650) linked to a Spectra-Physics SP4290 integrator.

Antisera against homogeneous preparations of pyruvate dehydrogenase complex and the E1 component of branched-chain 2-oxo acid dehydrogenase complex were raised in New Zealand White rabbits as described previously [20]. An immunoglobulin fraction was prepared by addition of $(\text{NH}_4)_2\text{SO}_4$ (60% satn. final concn.) to the antisera. After standing for 30 min, the immunoglobulins were collected by centrifugation (5 min at 10000 g), resuspended in 50 mM- $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$, pH 7.0, and then dialysed extensively against this buffer. Immunoprecipitates were prepared by incubating cell extracts with 10 μl of antiserum [or 2 μl of Ig (19 mg/ml)] for 15 min at room temperature and then at 4 °C overnight. Controls were incubated with normal rabbit serum instead of antisera. After incubation, 10 μl of Pansorbin was added, and the samples were mixed and left at room temperature for 15 min. After centrifugation for 2.5 min at 10000 g the supernatant was discarded and the immunoprecipitate washed four times with buffer containing 50 mM-Tris/HCl (pH 7.5), 0.9% (w/v) NaCl, 0.1% SDS and 1.0% Triton X-100. The immunoprecipitates were dissociated by boiling in 50 μl of Laemmli sample buffer, and then the phosphorylated protein was analysed by SDS/polyacrylamide-gel electrophoresis. After staining for protein, the dried gel was autoradiographed for 3–7 days. Phosphorylation of individual sites on the α -subunit of branched-chain 2-oxo acid dehydrogenase was analysed by tryptic digestion, high-voltage electrophoresis and autoradiography [12,13] of an excised band from a preparative gel.

Carrier-free [^{32}P]P_i was from Amersham International. Collagenase (type II), bovine serum albumin (essentially fatty acid-free) and branched-chain 2-oxo acids were from Sigma Chemical Co. Pansorbin was from Calbiochem. Branched-chain 2-oxo acid dehydrogenase complex was purified from bovine kidney cortex [11] and pyruvate dehydrogenase complex from bovine heart [21]. They were phosphorylated with [γ - ^{32}P]ATP as described in [12]. Trypsin was from Worthington Biochemicals.

RESULTS AND DISCUSSION

When isolated adipocytes are incubated in the presence of [^{32}P]P_i and the resultant phosphopeptides analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography, several major phosphopeptides are detected (Fig. 1a). The identity of several of these phosphoproteins has been established previously [22]. In particular the phosphoprotein of M_r 41000 has been identified as the α -subunit of pyruvate dehydrogenase complex [22] and

Fig. 1. Analysis by SDS/polyacrylamide-gel electrophoresis and autoradiography of phosphoprotein pattern of adipocyte extracts

Samples were prepared and analysed as described in the Materials and methods section. (a) Track 1, adipocyte extract; track 2, purified bovine kidney branched-chain 2-oxo acid dehydrogenase complex, phosphorylated with [γ - ^{32}P]ATP; track 3, purified bovine heart pyruvate dehydrogenase complex, phosphorylated with [γ - ^{32}P]ATP. (b) Track 1, adipocyte extract treated with control serum (Ig); track 2, immunosupernatant of extract after treatment with antibody (Ig) raised against branched-chain 2-oxo acid dehydrogenase (E1). A–D represent the positions of ATP citrate lyase, hormone-sensitive lipase, α -subunit of branched-chain 2-oxo acid dehydrogenase and α -subunit of pyruvate dehydrogenase respectively.

the phosphoprotein of M_r 46000 suggested as being the α -subunit of branched-chain 2-oxo acid dehydrogenase, based on its relative molecular mass and the observed effect of 4-methyl-2-oxopentanoate on its phosphorylation [17]. Comparison of the mobility of these phosphopeptides on SDS/polyacrylamide-gel electrophoresis with purified phosphorylated pyruvate dehydrogenase and branched-chain 2-oxo acid dehydrogenase (Fig. 1a) further substantiates their identification. However, availability of antiserum against the E1 component of branched-chain 2-oxo acid dehydrogenase allows the identity of the M_r -46000 phosphopeptide to be established unequivocally.

Fig. 2 shows that antiserum raised against the E1 component of bovine kidney branched-chain 2-oxo acid dehydrogenase selectively precipitates the M_r -46000 phosphopeptide from adipocyte extracts. Immunoprecipitation of the phosphoprotein is blocked in a competitive manner by the presence of increasing amounts of non-radioactive branched-chain 2-oxo acid dehydrogenase complex, but it is unaffected by the presence of equivalent amounts of pyruvate dehydrogenase complex (Fig. 2). Antiserum prepared against pyruvate dehydrogenase complex does not precipitate the phosphoprotein

Fig. 2. Immunoprecipitation of phosphoproteins from adipocyte extracts

Immunoprecipitates were prepared by using respective antisera, as described in the Materials and methods section, and analysed by SDS/polyacrylamide-gel electrophoresis and autoradiography. Tracks 1-7 and 9 show extracts treated with antiserum against E1 of branched-chain 2-oxo acid dehydrogenase: track 1, 5 μ l of antiserum; track 2, 10 μ l of antiserum; track 3, 20 μ l of antiserum; tracks 4, 5, 6 and 7, 10 μ l of antiserum in the presence of 5, 10, 25 and 50 μ g respectively of unlabelled branched-chain 2-oxo acid dehydrogenase complex; track 9, 10 μ l of antiserum in the presence of 50 μ g of unlabelled pyruvate dehydrogenase complex; track 10, extract treated with 10 μ l of antiserum against pyruvate dehydrogenase complex; tracks 8 and 11, purified phosphorylated branched-chain 2-oxo acid dehydrogenase and pyruvate dehydrogenase complexes respectively.

of M_r 46000, but, as expected, this antiserum precipitates the phosphopeptide of M_r 41000. Thus the phosphoprotein of M_r 46000 has been firmly established as the α -subunit of branched-chain 2-oxo acid dehydrogenase complex.

When the phosphoprotein of M_r 46000 is extracted from an SDS/polyacrylamide gel of adipocyte extract and the phosphorylation sites are then analysed, it can be seen (Fig. 3) that two major phosphopeptides are present, containing the two known phosphorylation sites on branched-chain 2-oxo acid dehydrogenase [13]. Furthermore two minor phosphopeptides, designated T1U and T2, are also derived from the same phosphorylation sites. It can therefore be concluded that the same two sites on the polypeptide are phosphorylated in intact adipocytes as are phosphorylated in isolated mitochondria [7] and by using purified enzyme [13]. Furthermore the presence in the tryptic digest of essentially only phosphopeptides derived from the α -subunit (Fig. 3) indicates that the band of radioactivity corresponding to M_r 46000 contains the α -subunit of branched-chain 2-oxo acid dehydrogenase as the major phosphorylated species. This is supported by

Fig. 3. Phosphopeptide analysis of phosphorylated branched-chain 2-oxo acid dehydrogenase

Samples were analysed by high-voltage electrophoresis and autoradiography as described in the Materials and methods section. Track 1, sample phosphorylated in adipocytes in control incubation. Track 2, sample phosphorylated in adipocytes incubated in presence of 2.5 mM 4-methyl-2-oxopentanoate. Samples in tracks 1 and 2 were obtained from an equal number of cells. Track 3, sample of purified branched-chain 2-oxo acid dehydrogenase. Phosphopeptides T1 and T3 contain phosphorylation sites 1 and 2 respectively. Phosphopeptides T1U and T2 are partial digestion products, containing the same two sites [13].

the finding that, after immunoprecipitation of the branched-chain 2-oxo acid dehydrogenase α -subunit, there is a marked disappearance of the phosphopeptide from the immunosupernatant (Fig. 1b).

From work with purified complex, intact mitochondria and perfused hearts [4,23,24] it has been established that the branched-chain 2-oxo acids inhibit branched-chain 2-oxo acid dehydrogenase kinase. Furthermore leucine and 4-methyl-2-oxopentanoate have been shown to activate the complex in adipose-tissue segments, consistent with inhibition of the kinase [25]. We have therefore investigated the effects of these substrates on the extent

Fig. 4. Densitometric scans of phosphoprotein patterns of adipocyte extracts

(a) Extract prepared from adipocytes from a control incubation; (b) extract from adipocytes incubated in the presence of 2.5 mM 4-methyl-2-oxopentanoate. A–D represent the positions of the reference proteins given in Fig. 1.

of phosphorylation of the complex within intact adipocytes. Fig. 4 shows that incubation of adipocytes in the presence of 4-methyl-2-oxopentanoate causes a significant decrease in the extent of labelling of the complex. A similar inhibition is observed with the other two branched-chain 2-oxo acids. The effect is specific for the α -subunit of the complex. No consistent effects are observed on the phosphorylation of any other phosphopeptide, although in some experiments an increase in the labelling of the α -subunit of pyruvate dehydrogenase is observed. The decrease in phosphorylation of the branched-chain 2-oxo acid dehydrogenase can be

observed within 2 min of addition of the 2-oxo acid but is maximal after 30–45 min (results not shown). The effect is dependent on the concentration of the 2-oxo acid, with decreased phosphorylation being observed in the presence of as little as 25 μ M substrate (Table 1), this concentration being well within the range found in normal plasma [26]. Analysis of the phosphopeptide pattern indicates that the phosphorylation of both sites on the α -subunit is decreased by the presence of 2-oxo acid, the effect on site 1 being more pronounced than that on site 2 (Fig. 3).

The data reported in the present paper further substantiate the role of phosphorylation as a regulatory mechanism for branched-chain 2-oxo acid dehydrogenase. Development of methods for study of the phosphorylation state of the complex will allow use of the isolated adipocytes as a model system for study of possible acute hormonal effects on phosphorylation of the complex.

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REFERENCES

1. Randle, P. J., Fatania, H. R. & Lau, K. S. (1984) *Mol. Aspects Cell. Regul.* **3**, 1–26
2. Pettit, F. H., Yeaman, S. J. & Reed, L. J. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4881–4885
3. Parker, P. J. & Randle, P. J. (1978) *FEBS Lett.* **95**, 153–156
4. Hughes, W. A. & Halestrap, A. P. (1981) *Biochem. J.* **196**, 459–469
5. Parker, P. J. & Randle, P. J. (1980) *FEBS Lett.* **112**, 186–190
6. Odessey, R. (1980) *Biochem. J.* **192**, 155–163
7. Cook, K. G., Lawson, R. & Yeaman, S. J. (1983) *FEBS Lett.* **164**, 85–88
8. Patel, T. B. & Olson, M. S. (1982) *Biochemistry* **21**, 4259–4265
9. Fatania, H. R., Lau, K. S. & Randle, P. J. (1981) *FEBS Lett.* **132**, 285–288
10. Odessey, R. (1982) *Biochem. J.* **204**, 353–356
11. Lawson, R., Cook, K. G. & Yeaman, S. J. (1983) *FEBS Lett.* **157**, 54–58

Table 1. Effect of branched-chain 2-oxo acids on phosphorylation of α -subunit of branched-chain 2-oxo acid dehydrogenase in isolated adipocytes

Cells were incubated in the absence or presence of branched-chain 2-oxo acids for 30 min. Extent of phosphorylation of the α -subunit of branched-chain 2-oxo acid dehydrogenase was determined from the integrated area of the corresponding peak on an autoradiograph of an SDS/polyacrylamide gel. The peak areas corresponding to ATP citrate lyase and hormone-sensitive lipase were used as internal standards in each track, to correct for variation in loading. Results are shown as means $\pm$ S.E.M., for the numbers of observations in parentheses. Statistical significance was determined by paired Student's *t* test. *P < 0.05; **P < 0.01.

Addition (mM)		Decrease (% of control) in phosphorylation of α -subunit	
4-Methyl-2-oxopentanoate	(0.025)	15 $\pm$ 5*	(5)
	(0.25)	31 $\pm$ 6**	(5)
	(2.5)	51 $\pm$ 6**	(4)
3-Methyl-2-oxobutyrate	(2.5)	25 $\pm$ 8*	(4)
3-Methyl-2-oxopentanoate	(2.5)	19 $\pm$ 4*	(4)

12. Cook, K. G., Lawson, R. & Yeaman, S. J. (1983) *FEBS Lett.* **157**, 59–62
13. Cook, K. G., Bradford, A. P., Yeaman, S. J., Aitken, A., Fearnley, I. M. & Walker, J. E. (1984) *Eur. J. Biochem.* **145**, 587–591
14. Waymack, P. P., DeBuysere, M. S. & Olson, M. S. (1980) *J. Biol. Chem.* **255**, 9773–9781
15. Harris, R. A., Paxton, R. & Parker, R. A. (1982) *Biochem. Biophys. Res. Commun.* **107**, 1497–1503
16. Buxton, D. B. & Olson, M. S. (1982) *J. Biol. Chem.* **257**, 15026–15029
17. Hughes, W. A. & Halestrap, A. P. (1980) *Biochem. Soc. Trans.* **8**, 374
18. Honnor, R. C., Dhillon, G. S. & Londos, C. (1985) *J. Biol. Chem.* **260**, 15122–15129
19. Laemmli, U. K. & Favre, M. (1973) *J. Mol. Biol.* **80**, 575–599
20. De Marcucci, O. & Lindsay, J. G. (1985) *Eur. J. Biochem.* **149**, 641–648
21. Stanley, C. J. & Perham, R. N. (1980) *Biochem. J.* **191**, 147–154
22. Hughes, W. A., Brownsey, R. W. & Denton, R. M. (1980) *Biochem. J.* **192**, 469–481
23. Lau, K. S., Fatania, H. R. & Randle, P. J. (1982) *FEBS Lett.* **144**, 57–62
24. Paxton, R. & Harris, R. A. (1984) *Arch. Biochem. Biophys.* **231**, 48–57
25. Frick, G. P., Tai, L.-R., Blinder, L. & Goodman, H. M. (1981) *J. Biol. Chem.* **256**, 2618–2620
26. Hutson, S. M. & Harper, A. E. (1981) *Am. J. Clin. Nutr.* **34**, 173–183

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