Vol. 105, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1982 Pages 705-710

MULTIPLE PHOSPHORYLATION OF RAT LIVER 3-HYDROXY 3-METHYLGLUTARYL COENZYME A REDUCTASE

Elisabet Font, Mercedes Sitges and Fausto G. Hegardt

Department of Biochemistry, University of Barcelona
School of Pharmacy, Barcelona-28 (Spain)

Received February 11, 1982

Rat liver homogeneous ^{32}P-labeled hydroxy methylglutaryl coenzyme A reductase, was treated independently with CNBr and trypsin and the resulting [^{32}P]phosphopeptides were analyzed by disc gel electrophoresis. CNBr treatment produced only one ^{32}P-fragment of Mr 18,000. The time course of trypsin hydrolysis initially showed the appearance of some phosphopeptides, which were lately converted in two phosphopeptides of low Mr. These results provide direct support for the concept that hydroxy methyl glutaryl coenzyme A reductase kinase solubilized from microsomes phosphorylates only two sites or set of sites in the reductase molecule.

Interconversion of enzymes by covalent modification is being increasingly recognized as a widespread mechanism of metabolic control (1-3). The covalent modification of rat liver 3-hydroxy 3-methylglutaryl coenzyme A reductase (EC 1.1.1.34) through a phosphorylation-dephosphorylation cycle, was demonstrated by us when we showed that the incorporation of ^{32}P from [γ-^{32}P] ATP into microsomal reductase (4,5) was a process closely linked to the inactivation of the enzyme. Similar findings were obtained by Beg et al (6) and Keith et al (7). Moreover, we reported (8) the characterization of a HMG-CoA reductase phosphatase, able to remove ^{32}P from homogeneous ^{32}P-labeled HMG-CoA reductase with a concomitant increase in reductase activity.

The important question as to whether the incorporation of phosphate groups involves a single or multiple sites in the subunit of reductase has not been studied. Keith et al (7) using [γ-^{32}P] ATP with an extremely low specific radioactivity (63 dpm/pmol) indicated that the level of phosphorylation of the enzyme was between 1 and 4 moles per mol of tetramer reductase. Somewhat

Abbreviations: HMG-CoA, 3-hydroxy 3-methylglutaryl coenzyme A; reductase, HMG-CoA reductase; $NaDodSO_4$, sodium dodecyl sulphate.

0006-291X/82/060705-06$01.00/0

more precise results were presented by Beg et al (9) who reported 3.68 moles phosphate per mol of reductase. However, the localization of sites in the subunit of reductase in which phosphate groups were attached, was not presented in those studies.

In this report, we present evidence for the multiple phosphorylation sites in reductase which decrease enzyme activity. Under the conditions used in this work, microsomal HMG-CoA reductase kinase phosphorylates two sites or set of sites in the reductase molecule.

MATERIAL AND METHODS

Chemicals Many reagents used in this work have been reported previously (8). Sodium dodecyl sulphate, acrylamide and bisacrylamide were from BioRad. Cyanogen bromide and urea were from Merck. N,N,N´,N´ tetramethylethylen diamine, soybean trypsin inhibitor, 2,5 diphenyloxazol and myoglobin were from Sigma. [γ-^{32}P]ATP (2,280 cpm/pmol) was from Amersham. Tosyl-L-phenylalanylchloromethane-treated trypsin was from Worthington. Highly radioactive (4.63 μCi/mg protein) homogeneous ^{32}P-labeled hydroxy methylglutaryl coenzyme A reductase was prepared as described in (10) using 0.4 mM [γ-^{32}P]ATP .

Assays The assay of HMG-CoA reductase has been described in (8). The assay of protein phosphorylation was done according to the method proposed by Huang and Robinson (11).

Analysis of phosphopeptides of HMG-CoA reductase

1. Cyanogen bromide treatment Aliquots of homogeneous ^{32}P-labeled HMG-CoA reductase (7-30 μg) were precipitated with equal volumes of 20% trichloroacetic acid at 0 °C and centrifuged at 1500 x g for 30 min. Pellets were dissolved in a (1:10 w/v) solution of CNBr in 70% formic acid at the proportion CNBr/reductase 500:1 (w/w). Samples were sealed, incubated at 25 °C for 24 h (12) and lyophylized. The CNBr peptides were dissolved by adding 50 μl of 10 M urea, 15 μl of sample buffer (40 mM sodium phosphate, 4 g/l $NaDodSO_4$, pH 6.8) and 60 μl marker solution (100 mM sodium phosphate, 50% glycerol and 4 g/l $NaDodSO_4$, pH 6.8) and boiled at 100 °C for 5 min. The CNBr peptides were subjected to disc gel electrophoresis on 8% polyacrylamide gels (0.5 x 15 cm) (13). After electrophoresis, gels were cut into 2 mm slices and radioactivity was quantitated with a liquid scintillation counter, taking advantage of the Cerenkov effect. Recovery of ^{32}P on the gels was 60-70% of the ^{32}P initially introduced into the HMG-CoA reductase. A parallel gel was run with the CNBr fragments of myoglobin Mrs 2510, 6210, 8160, 14400 and 16950.

2. Trypsin treatment Other aliquots of homogeneous ^{32}P-labeled HMG-CoA reductase were treated with trypsin as follows: Samples of reductase were lyophylized, the residues resuspended in 1 ml of 0.15 M NH_4CO_3H, pH 8.0 and trypsin at a ratio trypsin/protein 1:1 (w/w) was added. Samples were incubated at 37 °C for several periods ranging from 5 min to 60 hours repeating the addition of the same amount of trypsin every 12 hours. At indicated times, aliquots were removed and soybean trypsin inhibitor at a ratio 50:1 (w/w) with respect to trypsin was added, and the samples boiled at 100 °C for 5 min, frozen and lyophylized. The residue was resuspended in the same solution used for the

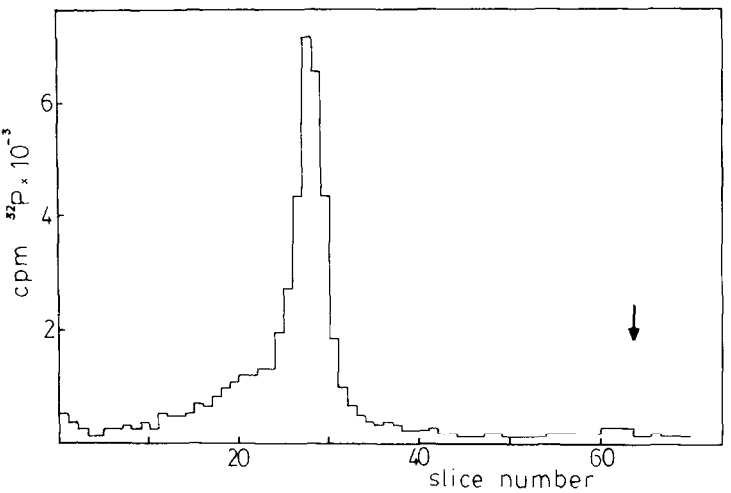

Figure 1 Electrophoretic analysis of CNBr phosphopeptides of homogeneous ^{32}P-labeled HMG-CoA reductase phosphorylated by endogenous microsomal reductase kinase. [^{32}P] HMG-CoA reductase obtained as described in (10) and containing 1.2 moles phosphate/mol enzyme was treated with CNBr, the resulting peptides were subjected to electrophoresis on 8% acrylamide $NaDodSO_4$-urea gels, as described in Methods. Gels were cut into 2 mm slices and radioactivity was determined using the Cerenkov effect. Slice 1 corresponds to the top of the gel and the arrow indicates migration of bromophenol blue

CNBr treatment and subjected to disc gel electrophoresis as described above but with 12% acrylamide. After running, gels were sliced and analyzed for radioactivity after addition of 2.5 ml of 5 g/l of 2-5 diphenyl oxazol in 2:1 (v/v) toluene-triton X-100. Recovery of ^{32}P on the gels was 50-60% of that present in HMG-CoA reductase used for trypsinization.

RESULTS

The results of the electrophoretic separation of phosphopeptides deriving from CNBr fragmentation of homogeneous HMG-CoA reductase, which had been inactivated by phosphorylation with [γ-^{32}P] ATP, leaving a 16% of total activity, is shown in Fig 1. There is only one peak of radioactivity with a mobility of approximately 0.40 on 8% acrylamide $NaDodSO_4$-urea gels and an apparent Mr of 18,000.

Since the Mr of CNBr [^{32}P]-phosphopeptide was rather high, and more than one phosphorylation site could be present in that peptide, a time course of trypsin fragmentation of ^{32}P-labeled HMG-CoA reductase was done. Samples were treated with trypsin at 5, 10, 30 min and 1, 2, 6, 12, 24, 36 and 60 hours and phosphopeptides separated as described in Methods.

Fig 2A represents the profile of phosphopeptides when incubation time was 5 min. Four different peaks appeared at Rf's 0.19

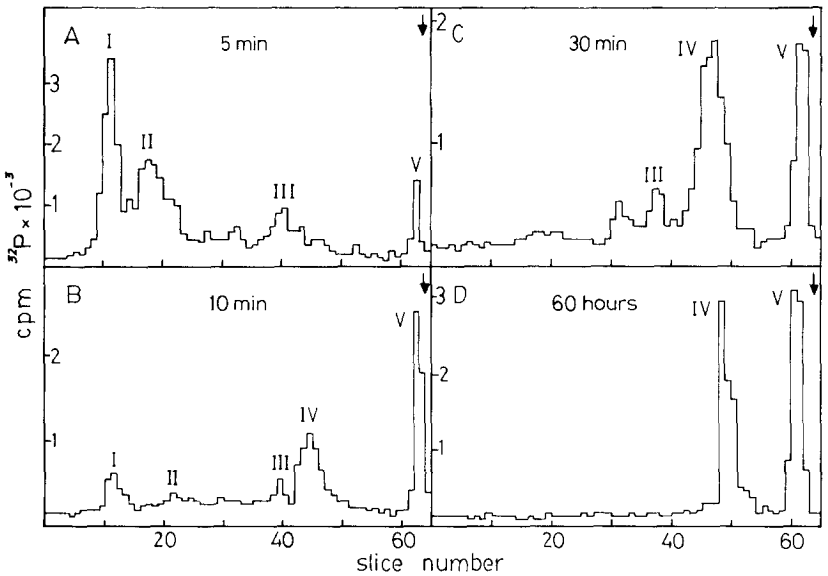

Figure 2 Electrophoretic analysis of tryptic phosphopeptides of homogeneous ^{32}P-labeled HMG-CoA reductase phosphorylated by endogenous microsomal reductase kinase. ^{32}P-labeled HMG-CoA reductase was treated with trypsin at different times of incubation, and the resulting peptides were subjected to electrophoresis on 12% acrylamide NaDodSO$_4$-urea. The distribution of ^{32}P-labeled phosphopeptides is shown. Panels A, B, C and D represent incubation times of 5, 10, 30 min and 60 hours, respectively. The Roman numerals identify what are discussed as peaks I to V in the text.

0.30, 0.66 and 0.96 and were called respectively peaks I, II, III and V. Peaks I and II corresponding to highest Mr, showed the highest radioactivity. An increase in the time of trypsin incubation up to 10 min produced a complete different pattern of phosphopeptides (Fig 2B). Peaks I, II and III showed less radioactivity compared with those of 5 min incubation; moreover, a new phosphopeptide at an Rf 0.72 (peak IV) appeared, and peak V increased.

When the incubation time was extended to 30 min (Fig 2C), peaks I and II completely disappeared, and peak III was very small whereas peaks IV and V were maximal. This pattern was analogous to those obtained at incubations for 1, 2, 6, 12, 24 and 36 hours (not shown). Finally, when ^{32}P-labeled reductase was incubated with trypsin for 60 hours (Fig 2D), peak III completely disappeared but peaks IV and V did not change. Molecular weights of these two peaks were not determined because they were beyond the calibration of gel, suggesting relatively small peptides.

DISCUSSION

Previous work (5,7,9) had reported the incorporation of ^{32}P into reductase only as a proof of the interconvertible character

of the enzyme. Preparation of highly radioactive ^{32}P-labeled HMG-CoA reductase (10), has led us to the study of phosphorylation sites in reductase molecule, since the analysis of phosphopeptides requires relatively high specific radioactivity of ^{32}P bound to the enzyme. Insofar, no other has reported the preparation of highly radioactive homogeneous ^{32}P-labeled HMG-CoA reductase; then it is not perhaps strange that no results have been reported on the number of sites able to be phosphorylated in HMG-CoA reductase.

In this report, we present evidence of the existence of at least two sites of phosphorylation in HMG-CoA reductase when the enzyme is phosphorylated by HMG-CoA reductase kinase from microsomes, up to 1.2 moles phosphate per mol reductase (Mr 200,000), what promotes an inactivation of 84%.

The time course of tryptic hydrolysis showed that at lower times of incubation, several phosphopeptides of high Mr appeared, suggesting that an incomplete tryptic cleavage had taken place; however, at 30 min incubation apparently the hydrolysis had almost finished. Two major peaks of low Mr are clearly seen together with a third minor peak which remains for 36 hours of incubation. However, the low level of radioactivity of the minor peak compared with those of major peaks, and its disappearance with a prolonged time of incubation, suggests that solubilized microsomal reductase kinase is responsible for phosphorylation in only two sites. Results obtained after CNBr treatment allow us to conclude that both sites of phosphorylation belong to the peptide of 18,000 daltons isolated after gel electrophoresis.

More detailed study of the relationship between phosphorylation in each site and kinetic properties will be of obvious importance in the future.

ACKNOWLEDGMENTS

This work was supported by a grant of Comisión Asesora de Investigación Científica y Tecnica and by L´Ajut a la Investigació de la Universitat de Barcelona, Spain

REFERENCES

1. Stadtman, E.R. and Chock, P.B. (1978) Curr Top Cell Regul 13, 53-95
2. Krebs, E.G. and Beavo, J.A. (1979) Annu Rev Biochem 48, 923-959
3. Cohen, P. (1980) Recently discovered systems of enzyme regulation by reversible phosphorylation. Elsevier Amsterdam
4. Bové, J. and Hegardt, F.G. (1978) FEBS Lett 90, 198-202

5. Gil, G., Sitges, M., Bové, J. and Hegardt, F.G. (1980) FEBS Lett 110, 195-199
6. Beg, Z.H., Stonik, J.A. and Brewer, H.B. (1978) Proc Natl Acad Sci USA 75, 3678-3682
7. Keith, M.L., Rodwell, V.W., Rogers, D.H. and Rudney, H. (1979) Biochem Biophys Res Commun 90, 969-975
8. Gil, G., Sitges, M. and Hegardt, F.G. (1981) Biochim Biophys Acta 663, 211-221
9. Beg, Z.H., Stonik, J.A. and Brewer, H.B. (1980) J Biol Chem 255, 8541-8545
10. Gil, G., Sitges, M. and Hegardt, F.G. (1981) Arch Biochem Biophys 210, 224-229
11. Huang, K.P. and Robinson, J.C. (1976) Anal Biochem 72, 593-599
12. Gross, E. (1967) Methods Enzymol 11, 238-255
13. Swank, R.T. and Munkries, K.D. (1971) Anal Biochem 39, 463-477