THE INHIBITORY EFFECT OF ATP ON HMGCoA REDUCTASE

Jacob C. Chow, Malcolm J. P. Higgins and Harry Rudney

Department of Biological Chemistry, College of Medicine
University of Cincinnati, Cincinnati, Ohio 45267

Received February 18,1975

SUMMARY. The inhibitory effect of ATP on HMGCoA reductase activity from rat liver microsomes in the system described by Beg et al. was examined. The inhibition by magnesium ATP is confirmed but varies widely from zero to complete. A requirement for a cytosolic fraction to enhance the inhibition could not be established. ATP labeled uniformly with ^{14}C in the adenine portion and ^{32}P in the terminal phosphate was incubated with the enzyme in a situation where strong inhibition was observed. The enzyme protein was precipitated with trichloroacetic acid, or subjected to column fractionation. No evidence of labeling was found in the protein. Finally, the enzyme protein was specifically isolated by immunoprecipitation with a specific antibody to the HMGCoA reductase. In no instance could labeling of the enzyme protein be detected. These results show that the mechanism of the inhibition does not involve phosphorylation or adenylation of the enzyme protein.

It has been widely accepted that HMGCoA reductase is the major locus of regulation of cholesterol biosynthesis from acetate (1,2). Recent work of Beg et al. has shown that rat hepatic microsomal HMGCoA reductase is inhibited by ATP and Mg^{++} (3). A rat liver cytosolic fraction was required as an adjuvant to this effect. In addition, cyclic AMP also appeared to enhance the inhibition. These results suggested the mechanism of inhibition by ATP might involve phosphorylation of the enzyme protein.

Abbreviations: HMGCoA, 3-hydroxy-3-methylglutaryl CoA; HMGCoA reductase, 3-hydroxy-3-methylglutaryl; CoA reductase, E.C. 1.1.1.34; cAMP, 3'5'-cyclic adenosine monophosphate.

The availability of a specific antibody to the rat liver reductase enabled us to readily test this concept. The work presented here describes experiments on the inhibition of HMGCoA reductase by ATP labeled with ^{32}P in the terminal phosphate and ATP with ^{14}C labeled uniformly in the adenine ring. Attempts to detect phosphorylation of the inhibited enzyme using immunoprecipitation to isolate the enzyme protein are also described.

Materials and Methods

Female rats (150-200 g) were obtained from Sprague-Dawley Inc., Madison, Wisconsin, and maintained in a twelve hour dark and twelve hour light cycle.

Isolation of the rat liver microsomes, and preparation of the cytosolic fraction by ammonium sulfate precipitation were carried out according to Beg *et al.* (3), except that the microsomal pellet was washed by resuspension in the same buffer, and centrifugation at 100,000 x g for one hour. The washing was repeated twice. The washed microsomal pellets were then used for ATP incubation experiments. Preparation of ^{14}C-HMGCoA and assays of HMGCoA reductase were carried out according to Higgins *et al* (4). Protein was determined according to Lowry *et al.* (5).

For ^{32}P- or ^{14}C-incorporation experiments, thrice washed rat liver microsomes were incubated at 37° for 20 min with 4 mM ATP which contained appropriate amounts of ^{32}P-ATP or ^{14}C-ATP or both, and 4 mM Mg^{++}. The microsomes were then

separated from the mixture by centrifugation at 100,000 x g for one hour and the supernatant fluid was discarded. The microsomal preparations were then washed twice as described previously. The microsomal pellets were then resuspended in buffer and an aliquot was used for ^{32}P determination. The remainder was solubilized according to the freeze-thaw procedure of Heller and Gould (6) and then used for immunological analysis and for partial purification of HMGCoA reductase. Fractions of partially purified enzyme were assayed for radioactivity. The solubilized crude extract was precipitated by specific antibody against HMGCoA reductase (4). The antigen-antibody precipitate was then washed four times with isotonic NaCl, suspended in aquasol and assayed for radioactivity in a scintillation spectrometer.

^{32}P-determination was carried out as follows: 0.1 ml of the microsomal suspension was added to 1.0 ml of cold 10% trichloroacetic acid and allowed to stand for 10 min in an ice bath. The precipitate was removed by centrifugation and dissolved in 1 N NaOH. The precipitation and solution procedures were repeated twice. The final suspension was assayed for ^{32}P utilizing aquasol as previously described.

Results and Discussion

The data obtained from a typical experiment are listed in Table I and show that incubation of rat liver microsomes with 4 mM ATP and Mg^{++} results in 60% inhibition of HMGCoA reductase activity. The addition of a cytosolic fraction obtained by

35-45% $(NH_4)SO_4$ fractionation appeared unnecessary and actually results in a decreased inhibitory effect.

Table I. Effect of ATP, Mg^{++} and a Cytosolic Fraction on HMGCoA Reductase

Experiments	Specific Activity nmole/mg/min
1.Control (microsomes only)	0.36
2. Microsomes + 4 mM ATP + Mg^{++}	0.15
3. Microsomes + 35-45% ammonium sulfate fraction	0.30
4. Microsomes + 35-45% ammonium sulfate fraction + 4 mM ATP + Mg^{++}	0.24

All incubations contained 2.7 mg of microsomes with 2.0 mg of 35-45% ammonium sulfate fraction samples 3 and 4 in a total volume of 1.0 ml.

In order to determine whether the ATP inhibition involved phosphorylation of the enzyme protein, ^{32}P-γ-ATP and ^{14}C-ATP were incubated with rat liver microsomes that had been washed three times and treated further as described in the Methods section. The results of a representative experiment are shown in Table II. Either ATP and Mg^{++} alone or with the cytosolic fraction could completely inhibit HMGCoA reductase activity. Washing of the inhibited microsomes increased enzyme activity (Expt. 4b). The microsomal suspensions were solubilized

Table II. Incubation of HMGCoA Reductase with Labeled ATP.

Sample	HMGCoA Reductase nmole/mg/min	Radioactivity dpm/mg protein: Soluble microsomal fraction	Radioactivity dpm/mg protein: CCl_3COOH precipitate
1. None	0.0930		
2. ATP + Mg^{++}	0*	^{32}P - 3920 ^{14}C - 2200	0* 0
3. ATP + Mg^{++} + cytosolic fraction	0	^{32}P - 3720 ^{14}C - 2150	0
4. ATP + Mg^{++} + cytosolic fraction	0.0110		
a. After 1 wash	0.0120		
b. After 2 washes	0.0320		
c. After solubilization	0.0100	^{32}P - 1775	0

* 0 indicates non-detectable enzymatic activity or radioactivity.

Tubes 1, 2 and 3 each contained 20 mg of microsomal protein, 4 mM ATP, 4 mM Mg^{++} in a total volume of 10 ml. Tube 3 contained 5 mg of 35-45% ammonium sulfate fraction of the cytosol. Tube 4 contained 400 mg of microsomes, 4 mM ATP, 4 mM Mg^{++} and 50 mg of cytosolic fraction in a total volume of 200 ml. The ATP in tubes 2 and 3 contained 4 microcuries of uniformly labeled ^{14}C ATP and 12.5 microcuries of ^{32}P-γ-ATP. The ATP in tube 4 contained 125 microcuries of ^{32}P-γ-ATP.

and assayed for radioactivity before and after solubilization. ^{32}P determination of the protein was carried out before and after solubilization, no ^{32}P activity was found in the proteins of three sets of incubations before and after solubilization. After two washings, ^{14}C and ^{32}P activity was associated with the microsomes, yet no radioactivity was detected in the TCA precipitatable protein of these samples. Furthermore, no

radioactivity was detected in the specific antigen-antibody precipitate of the solubilized microsomes assayed in Expts. 1 and 2. If ATP was indeed bound to the enzyme in the molar ratio of one to one, then based on enzyme protein present and molecular weight of the enzyme several hundred dpm of either ^{14}C or ^{32}P would be detected in the antigen-antibody complex.

In another experiment, HMGCoA reductase was solubilized and partially purified according to the method of Heller and Gould (Sample 4c, Table II) (6). Only ten tubes with radioactivity above background were detected from fractions eluted from the agarose column and these did not coincide either with the protein or enzyme peaks. The data listed in Table II suggest the enzyme protein was neither adenylated nor phosphorylated by ATP.

Our results have shown that ATP and Mg^{++} can inhibit rat liver microsomal HMGCoA reductase activity and a cytosolic fraction was unnecessary. We have also observed that 4 mM ATP and Mg^{++} inhibit HMGCoA reductase activity in rat liver microsomes which had been washed twice (7). This kind of inhibition has also been reported by Beg *et al.* (3) and Shapiro *et al.* (8). However, we did not observe effects of other cytosolic fractions on HMGCoA reductase activity when incubated with or without ATP. Also, we found that cyclic AMP could also inhibit HMGCoA reductase activity (7). It must be emphasized, however, that the inhibition with ATP is variable and not consistently reproducible and in our hands has ranged from zero to complete. However, the data here apply to situations where inhibition is

actually observed and in these cases the data show unequivocally that ATP inhibition is neither due to phosphorylation of the enzyme protein nor caused by covalent binding of ATP to enzyme protein. Preliminary results (7) suggest that competition between enzyme substrate and ATP might be responsible for the inhibition. The observations of Deal and collaborators (9) on the competitive inhibition of glyceraldehyde phosphate dehydrogenase by adenine nucleotides and cyclic AMP may be analogous to the reported effects of ATP and cyclic nucleotides on HMGCoA reductase.

Acknowledgement. This work was supported by a grant from the National Institute of Arthritis and Metabolic Diseases, AM-12463.

References

1. Rodwell, V. W., McNamera, D. J., and Shapiro, D. J. (1973). Advan. Enzymol. 38, 373.

2. Siperstein, M. D. (1970) in Current Topics in Cellular Regulation (Horecker, B. L. and Stadtman, E. R., ed.) Vol. 2, 65, Academic Press, N. Y.

3. Beg, Z. H., Allman, D. W., and Gibson, D. M. (1973). Biochem. Biophys. Res. Commun. 54, 1362.

4. Higgins, M. J. P., Brady, D., and Rudney, H. (1974). Arch. Biochem. Biophys. 163, 271.

5. Lowry, O. H. Rosenbrough, D. J., Farr, A. L., and Randall, R. J. (1951). J. Biol. Chem. 193, 165.

6. Heller, R. A. and Gould, G. (1973). Biochem. Biophys. Res. Commun. 50, 859.

7. Chow, T. C., Higgins, M. J. P., and Rudney, H. (1974). Unpublished data.

8. Shapiro, D. J., Nordstrom, J. L., Mitschelen, J. T., Rodwell, V. W., and Schimke, R. T. (1974). Biochim. Biophys. Acta 370, 369.

9. Yang, S. T. and Deal, W. C., Jr. (1969). Biochemistry 8, 2806.