# EFFECT OF A PEPTIDE STABILIZING FACTOR ON LIVER ATP CITRATE LYASE *

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SUMMARY: The activity of ATP citrate lyase, the enzyme responsible for production of cytoplasmic acetyl CoA for lipogenesis, is known to be modulated by dietary manipulation. The present work suggests a mechanism for control of the rate of its degradation. An unusual peptide factor, shown earlier by Dunaway and Segal [*J. Biol. Chem.* *251*, 2323-2329 (1976)] to stabilize phosphofructokinase from thermal or lysosomal degradation, exerts similar stabilizing effects on ATP citrate lyase. These experiments confirm that the effects of the stabilizing factor are pleitropic, and suggest that it may play a role in the control of turnover of other lipogenic enzymes whose activities are similarly modulated.

The function of ATP citrate lyase (EC 4.1.3.8) is to provide for the synthesis of acetyl CoA in the cytoplasm from citrate derived from mitochondria. It has been known for some time that the activity of this enzyme in liver, together with other key lipogenic enzymes, could be profoundly influenced by dietary or hormonal manipulation (1). For example, the activity of the enzyme has been observed to decline during fasting and then to be enhanced manyfold by subsequent consumption of a high carbohydrate diet (2). Observations such as these imply influence by insulin or glucagon on the rate of synthesis or turnover of the enzyme in liver.

Recently, Dunaway and Segal (3) have described an intriguing peptide factor found to be produced in mammalian liver following similar dietary manipulation. They found that the factor, of molecular weight near 3500, could protect the glycolytic enzyme phosphofructokinase from either thermal or lysosomal inactivation, implying that interaction of the enzyme with the stabilizing factor could account for its retarded intracellular degradation and turnover during refeeding. Experiments reported herein show that the stabilizing factor exerts similar protective effects on ATP citrate lyase *in vitro*, thus suggesting a pleiotropic effect for the factor.

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## MATERIALS AND METHODS

Treatment of Animals: Woodlyn/Wistar Rats (Woodlyn Lab., Guelph, Ontario) weighing about 180-200 g were used. For the purification of ATP citrate lyase, the rats were starved for two days and then refed high carbohydrate fat-free diet for three days. The stabilizing factor was obtained from livers of rats which had been starved for four days and then refed with the same diet for one day. The high carbohydrate fat-free diet contained 63% sucrose, 24% casein, 2.5% cellulose powder, salt and vitamin mixture.

Purification of ATP citrate lyase: ATP citrate lyase was purified according to a procedure adopted from Inoue *et al* (4) with the following modifications. Rat livers were homogenized in three volumes of 10 mM Tris-HCl pH 7.4 containing 200 mM KCl, 1 mM EDTA, 10 mM β-mercaptoethanol and 5 mM sodium citrate. Following centrifugation at 15000 x g for 40 min, 2% protamine sulfate was added to give a final concentration of 0.2%. After centrifugation, solid $(NH_4)_2SO_4$ was added to 45% saturation. The precipitate was dissolved in 10 mM Tris-HCl pH 7.8 containing 20 mM KCl, 1 mM EDTA, 10 mM β-mercaptoethanol and 5 mM sodium citrate and was dialyzed against the same buffer. DEAE-cellulose was equilibrated with the dialysis buffer and elution was carried out by using a continuous gradient of dialysis buffer adjusted to pH 7.4 and a final concentration of KCl of 400 mM. Active fractions were pooled and concentrated with solid $(NH_4)_2SO_4$ at 45% saturation. The precipitate was dissolved in homogenizing buffer and applied to a column of Sephadex G-200 equilibrated with the same buffer. Fractions with specific activity over 1.0 were pooled and sucrose was added to 1 M final concentration. The enzyme so obtained was stored at -20° until used.

Purification of stabilizing factor: The procedure of Dunaway and Segal (3) was followed up to and including the dialysis step, except that the dialysis buffer that was used was 50 mM Tris-HCl pH 8.3 containing 100 mM glucose and 10 mM β-mercaptoethanol. The stabilizing factor rapidly loses activity, so before proceeding to the experiment reported in Figs. 3 and 4 it was necessary to further concentrate the solution by treatment with Sephadex G-25 Coarse. This led to some loss of protein and the final concentration was measured to 37.9 µg/ml.

Assay of ATP citrate lyase: ATP citrate lyase was assayed by coupling to malate dehydrogenase and following the decrease in absorbance at 340 nm (5). The assay medium contained 20 mM sodium citrate, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 200 mM Tris-HCl pH 8.4, 10 mM ATP, 3 units malate dehydrogenase and 0.2 mM CoASH in a total volume of 0.52 ml. The reaction was initiated by addition of CoASH.

Thermal degradation studies: A 75 µl solution of ATP citrate lyase (2.1 mg/ml, 1.1 units/mg) was mixed with 75 µl stabilizing factor or buffer (50 mM Tris-HCl pH 8.3, 100 mM glucose and 10 mM β-mercaptoethanol). Immediately a sample was removed for assay, giving the activity at zero minutes. The remaining mixture was rapidly heated in a waterbath at 45°. When the temperature of the mixture reached 35° it was transferred to a Temp-Blok Module Heater (Lab-Line Instruments Inc., Melrose Park, Ill.) set at 37°. Samples were removed at intervals to be transferred immediately to medium for assay of ATP citrate lyase. The reaction was started within 20 s of removal of the sample.

Calculation of stabilizing effect: Each curve in Figs. 1 and 4 represents three separate experiments. The value of $t_{0.5}$ is the time in minutes needed for 50% inactivation. The stabilizing effect of the factor is measured as the increase in $t_{0.5}$ observed with the addition of the factor. A similar

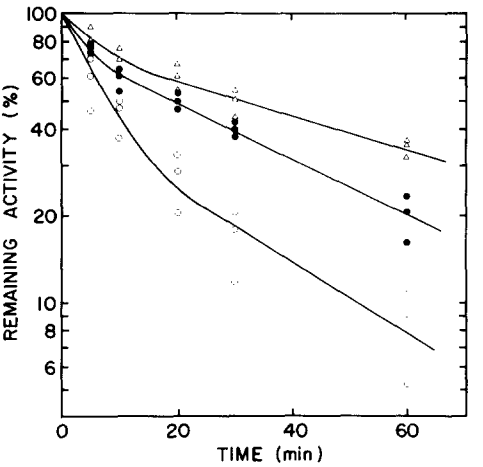


Fig. 1. Effect of stabilizing factor on the thermal denaturation of ATP citrate lyase. ATP citrate lyase was incubated at 37° with various amounts of stabilizing factor (87.9 µg/ml). For experimental details see "Materials and Methods". At each concentration, the rate of degradation in three separate incubation mixtures was determined (○) no stabilizing factor, (●) 50 µl stabilizing factor and (Δ) 75 µl stabilizing factor. An identical experiment with 25 µl stabilizing factor was omitted from the figure for the sake of clarity.

assay of factor activity was used by Dunaway and Segal (3) in their measurements of stabilization of phosphofructokinase.

Tryptic treatment of ATP citrate lyase: Tryptic digestion of ATP citrate lyase was done according to Singh *et al* (6). To 1 mg of enzyme was added 0.01 mg TPCK[1]-treated trypsin (Worthington Bioch. Corp.), and after 20 min incubation at 25° 0.4 mg TLCK was added to stop the reaction. Trypsin and TLCK were dissolved in 0.1 M Tris-HCl pH 7.4. In the control sample, buffer was added instead of trypsin.

Protein determination: Enzyme protein was measured according to Lowry *et al* (7) using bovine serum albumin as standard. Concentration of stabilizing factor was estimated from absorbance at 190 nm following exhaustive dialysis against water (3).

Polyacrylamide gel electrophoresis: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was done according to Weber and Osborn (8). Gels with half the normal mount of crosslinker were used.

## RESULTS

ATP citrate lyase, diluted with buffer to a concentration of 1.05 mg protein/ml and incubated at 37°, rapidly loses activity with an observed $t_{0.5}$ of 8 min (see Fig. 1). When stabilizing factor is added in increasing

[1] The abbreviations are : TPCK, L-1-Tosylamide-2-phenylethylchloromethyl ketone; TLCK, N-α-p-Tosyl-L-lysine chloromethyl ketone; SDS, sodium dodecyl sulfate.

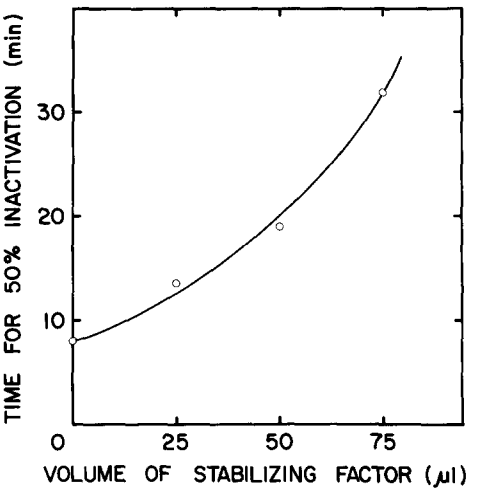


Fig. 2. The time for 50% inactivation from Fig. 1 was plotted against volume of stabilizing factor.

amounts to the enzyme and the mixture is incubated at 37° as before, it stabilizes ATP citrate lyase against thermal denaturation. The observed stabilization of ATP citrate lyase is a function of the concentration of factor (Fig. 2). Controls in which equivalent amounts of dialysate replaced the factor showed no stabilization.

Dunaway and Segal (3) have reported an unusual amino acid composition for the phosphofructokinase stabilizing factor. The ratio of glu:gly:$^{1}/_{2}$ cys was found to be 1.00:0.96:0.85 with only traces detected of other amino acids. We have performed amino acid analyses on acid hydrolysates of our stabilizing factor preparation and of a performate-oxidized sample. The results agree with those of Dunaway and Segal (3), indicating approximately equal amounts of glu, gly and 1/2 cys plus small (<5%) traces of other ninhydrin-positive material. This confirms the identity of our stabilizing factor preparation with that described for phosphofructokinase (3).

Recently, Singh *et al* (6) demonstrated that ATP citrate lyase, under controlled conditions, could be "nicked" by protease treatment giving rise to a fully active but relatively unstable derivative. This process appears

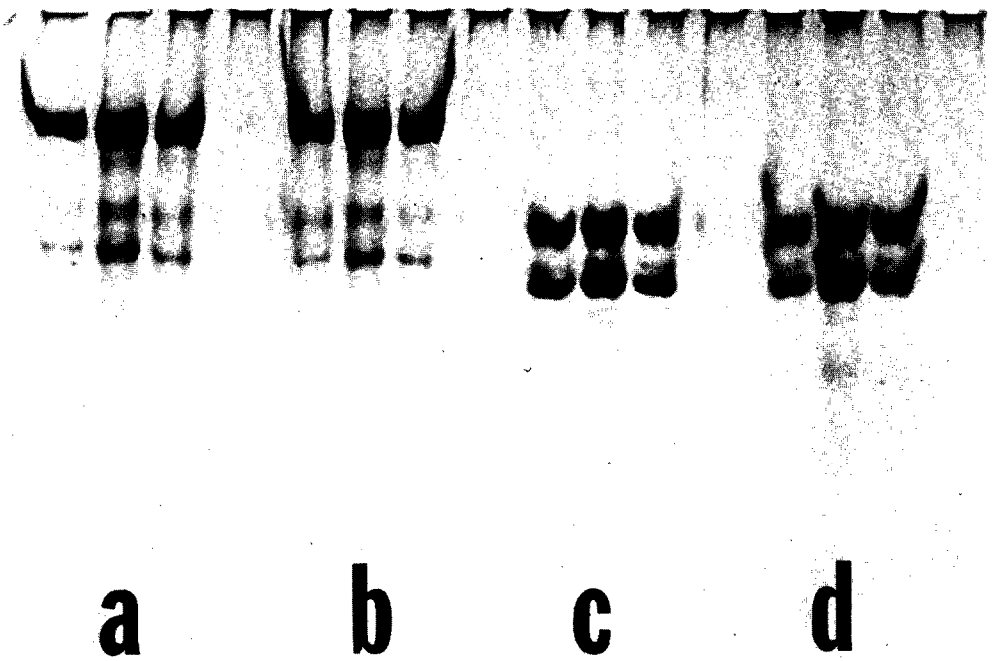


Fig. 3. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of native and trypsin treated ATP citrate lyase. 5 μg (outer tracks in each set of three) or 10 μg (middle track) of ATP citrate lyase were electrophoresed on 3 mm slabs in the presence of 0.1% sodium dodecyl sulphate and the protein was stained with Coomassie brilliant blue. The samples applied were a) native enzyme; b) enzyme carried through proteolysis procedure but without trypsin addition; c) trypsin treated enzyme; and d) enzyme treated with trypsin in the presence of stabilizing factor (see "Materials and Methods").

to involve cleavage of the 110,000 mol. wt. subunit to produce two smaller fragments. It was of obvious interest to determine the possible relationship of proteolysis to the effect of the stabilizing factor on the enzyme. Treatment of ATP citrate lyase with trypsin according to the method of Singh *et al* (6) resulted in conversion of the major peptide to two smaller fragments (Fig. 3), a process accompanied by no detectable loss in activity. When the "nicked" enzyme was incubated at 37°, it was rapidly inactivated with a $t_{0.5}$ of 3.8 min, confirming the increased lability described by Singh *et al* (6). When the "nicked" enzyme was incubated in the presence of stabilizing factors, we observed a degree of protection from inactivation comparable to that seen with native enzyme (Fig. 4). When the stabilizing factor is

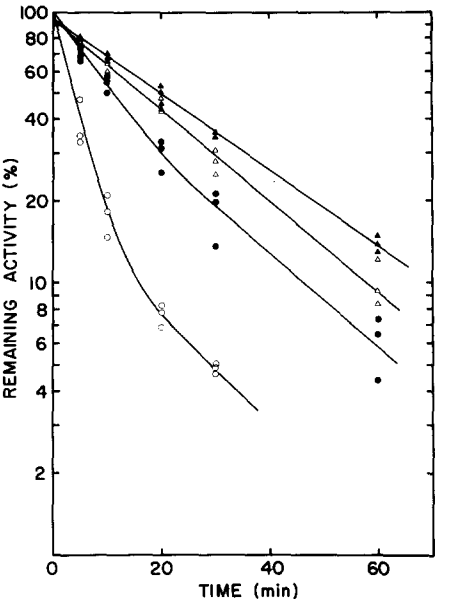


Fig. 4. Effect of stabilizing factor on nicked and unnicked ATP citrate lyase. The rate of degradation was measured in a mixture of 75 µl trypsin-treated enzyme plus (●) or minus (○) stabilizing factor (37.9 µg/ml) in a total volume of 150 µl at 37°. Δ—Δ: enzyme which had been pretreated with trypsin in the presence of stabilizing factor (see "Materials and Methods"). Stabilizing factor was present at equivalent concentrations during the inactivation incubation. ▲—▲: control in which enzyme was handled identically except that trypsin was omitted from the pre-treatment. Each experiment was done in triplicate.

present during the trypsin treatment, no change in the pattern of proteolysis was detected by SDS-polyacrylamide gel electrophoresis (Fig. 3); nevertheless, the resultant product is protected to a significantly greater extent than the enzyme which had been "nicked" in the absence of factor (Fig. 4). These results suggest that while the factor apparently does not prevent the major endoproteolytic cleavage, it may protect against proteolysis near the end of a polypeptide chain which may be accompanied by further reduction in stability but little change in size.

## DISCUSSION

The foregoing results indicate that a peptide factor produced in liver has the ability to confer thermal stability on the lipogenic enzyme ATP citrate lyase, and that the factor in question is very likely identical to

one previously shown to stabilize phosphofructokinase *in vitro*. Many intriguing questions are raised by these observations. Obviously, among these is the molecular mechanism of the stabilization process, elucidation of which will require knowledge of the structure of the factor and its target enzymes, together with an understanding of denaturation process. Such studies are presently underway in this laboratory.

A second question raised is whether the effects described above are of physiological significance, accounting at least in part for the observed burst of enzyme activity in livers of rats subjected to the appropriate dietary manipulation. We would not argue that decreased turnover is solely responsible for the burst, since controls on the rate of synthesis of the enzyme are likely to occur (9). In fact we have found in other experiments that administration of inhibitors of cyclic-AMP phosphodiesterase to animals during dietary manipulation effectively blocks the burst, suggesting an involvement of cyclic AMP in repression of synthesis.* However, for other enzymes of lipogenesis which are subject to similar dietary induction, namely malic enzyme (10) and acetyl CoA carboxylase (11-13), results have been presented consistent with independent controls upon both rate of enzyme synthesis and degradation. Also noteworthy in this context is the continuous requirement for a product of protein synthesis for the regulation of protein turnover in cultured hepatoma cells (14). All of this clearly raises a third question, to which we are presently addressing ourselves, the possibility that the stabilizing factor may interact not only with phosphofructokinase and ATP citrate lyase, but also with several other enzymes whose activity is known to be elevated in liver following dietary manipulation or insulin administration.

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* B. Osterlund and W.A. Bridger, unpublished observations.

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