

Full Length Research Paper

Modulation of partially purified rat liver mitochondrial carbamoyl phosphate synthetase I using two glutamic acid analogues

Shadrack Kamau Machua^{1*}, Joseph Chege Mukuria², Raphael Muchangi Ngure¹
and Peter M. Gitu³

¹Department of Biochemistry, Egerton University, P. O. Box 536-20115, Egerton, Kenya.

²Department of Biochemistry, University of Nairobi, P. O. Box 30197-00100, Nairobi, Kenya.

³Department of Chemistry, University of Nairobi, P. O. Box 30197-00100, Nairobi, Kenya.

*Corresponding author. E-mail: machuask@gmail.com. Tel: +254 722 678760, 733 754504.

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Mitochondrial carbamoyl phosphate synthetase I (CPS I) is the first enzyme involved in urea biosynthesis in ureotelic mammals and has an absolute requirement for N-acetyl-L-glutamate (NAG) or N-carbamyl-L-glutamate (NCLG) in absence of NAG as its allosteric modulator. To investigate effect of diet on CPS I activation, three male albino rats were maintained under normal laboratory diet (control) and another three on high protein egg white diet for 10 days. The percentage mean weight gain for the normal diet was 6.4% while the percentage mean weight loss for the high protein diet group 18.6%. The rats were sacrificed and CPS I isolated from the liver mitochondria through differential centrifugation and partially purified by ammonium sulphate precipitation, gel filtration on Sephadex G-200 and native polyacrylamide gel electrophoresis (PAGE). Lactate dehydrogenase-pyruvate kinase (LDH-PK) coupled assay system was devised to determine the effect of NAG and its structural analogue NCLG in the activation of CPS I from rats fed on the two diets. CPS I activity of 268.16 nmol/min/mg in the control group doubled to 553.86 nmol/min/mg in rats fed on high protein. An initial velocity of CPS I of 3.07 nmol/min/mg was observed when activated by 0.57 mM NAG and a lowered activity of 2.2 nmol/min/mg when replaced with 0.57 mM NCLG. Both NAG and NCLG activated CPS I at all concentrations tested in the assay system devised with improved activity seen when CPS I activity was measured in presence of NAG.

Key words: Modulation, carbamoyl phosphate synthetase I, N-acetyl-L-glutamate, N-carbamyl-L-glutamate, coupled assay, hyperammonemia.

INTRODUCTION

Carbamoyl phosphate synthetase I (CPS I) is urea cycle regulatory enzyme with an obligatory requirement for N-acetyl-L-glutamate (NAG) as its positive allosteric modulator (Caldovic and Tuchman, 2003). The reaction catalysed by CPS I is the first and rate limiting in mammalian ureagenesis (Haskins et al., 2008) occurring

to a great extent within periportal hepatocytes of the liver while low levels occurs within the enterocytes (Morris Jr., 2002). CPS I is a heterodimer composed of a 40 kDa and 120 kDa subunits (Ozaki et al., 1995). Lusty (1978) found the concentration of CPS I protein to be approximately 20% of mitochondrial matrix protein and the

concentration is directly related to intra-mitochondrial concentration of its substrate ammonia and of its activators NAG and free Mg^{2+} ions (Lof et al., 1983). Mammalian NAG, synthesized by the mitochondrial enzyme N-acetyl-L-glutamate synthetase (NAGS) (Elpeleg et al., 2002) is the naturally occurring allosteric activator of CPS I in ureotelic animals (Marshall et al., 1961). It has been reported that without NAG the enzymatic activity of CPS I is practically undetectable (Caldovic and Tuchman, 2003). CPS I exists in both monomeric and dimeric forms while the interaction with NAG causes conformational change in CPS I protein to yield a catalytically active but unstable configuration that is mainly monomeric (Lusty, 1981). Inherited NAGS deficiency an autosomal recessive disorder affecting urea synthesis has been reported (Elpeleg et al., 2002; Caldovic and Tuchman 2003) and causes severe hyperammonemia in neonates (Gessler et al., 2010) or later in life (Häberle, 2011). N-carbamyl-L-glutamate (NCLG) a structural analogue of NAG substitutes NAG in the activation of CPS I in disease states involving patients with inborn errors in NAGS gene (Danioi et al., 2011) although with lower affinity for CPS I (Rubio and Grisolia, 1981). Further it was demonstrated that NCLG administered together with arginine reduces blood ammonia, increase blood urea and protect animals administered a lethal dose of ammonia (Grau et al., 1992). In human, NCLG is used for the treatment of hyperammonemia due to NAGS deficiency (Tuchman, 2005; Häberle, 2011). The goal of this study was to investigate the effect NAG and NCLG in the activation of partially purified rat liver mitochondrial CPS I in a spectrophotometric coupled assay method involving lactate dehydrogenase (LDH) and pyruvate kinase (PK).

MATERIALS AND METHODS

Chemicals

The laboratory chemicals used in all experiments were of analytical grade and were obtained from Sigma Chemical Company (St. Louis, USA), Bio-Rad (Richmond USA), Pharmacia and Diagnostic Chemicals (Uppsala Sweden), Aldrich Fine Chemicals, UK and Fluka Fine Chemicals. All other reagents used in this study were supplied by Kobian Kenya Limited.

Experimental animals

Male Albino rats weighing between 250-350 g in body weights were randomly selected, weighed and maintained in the animal house at the Department of Biochemistry, University of Nairobi (UoN). The rats were divided into two groups each made of three rats. One group was fed on normal laboratory diet of commercial

pellets supplemented with vegetables (control). The second group of rats were placed in a separate cage, starved for 24 h and fed ad libitum on a diet containing high protein composed of 10 g of egg white protein per 100 g body weight for 10 days prior to sacrifice as described by Tujioka et al. (2005).

Preparation of mitochondria

The preparation of liver homogenates was performed as described in a modified method of Lusty (1978). The experimental rats were fasted overnight, sacrificed by decapitation and thoroughly exsanguinated. For each rat, the liver tissues were quickly excised and transferred to cold 0.25 M sucrose, pH 7.0 at 4°C. After blotting with tissue and weighing, 200-500 mg of the tissue were homogenized with a Teflon homogenizer for 45 s at 800 rpm in 20 volumes of cold 0.25 M sucrose, pH 7 (Lusty, 1978). Rat-liver mitochondria were isolated from the crude homogenate in a modified method by Myers and Slater (1957). The liver homogenate was centrifuged for 10 min at 2,000 rpm. Approximately three-quarters of the supernatant was decanted, the pellets re-homogenised and re-centrifuged as before. Further differential centrifugation of the supernatant was done for 10 minutes at 5,500 rpm, 12,000 rpm for 15 min and at 18,000 rpm for 30 min collecting supernatant of each step. All the centrifugation steps were carried out at 4°C in a refrigerated centrifuge. Glutamate dehydrogenase activity was used as marker for the mitochondrial fraction as in the method by Engel and Palziel (1970).

Preparation of CPS I from the rat liver mitochondria

An aliquot of mitochondria suspended in 0.25 M sucrose solution was lysed with 1 % Triton X-100 and to release CPS I into the solution (Lusty, 1978) and centrifuged at 12,500 rpm for 10 min in plastic falcon tubes as described by Rajiman and Bartulis (1979). The supernatant was decanted for analysis of CPS I and purification.

CPS I partial purification

Ammonium sulphate precipitation

To the supernatant obtained as described previously, solid ammonium sulphate was added gradually to a final concentration of 19.8 g/100 ml (35%), 8.9 g/100 ml (50%) and 39.3 g/100 ml (80%) with gentle stirring for 30 min at 4°C as described in modified method by Corvi et al. (2001). The suspension was centrifuged at 20,500 rpm for 20 min and the pellets suspended in 3 ml of 0.3 M K_2HPO_4 buffer, pH 7.6 and then dialysed overnight

against the same buffer.

Gel filtration

The 80% ammonium sulphate mitochondrial matrix protein suspension previously stored at -20°C was thawed at 4°C and loaded on sephadex G-200 gel filtration column (200-300 mesh particle size) in a modified method described by Andrews (1965). The slurry was suspended in phosphate buffer, pH 7.6 and allowed to pack into the column with internal diameter of 1.42 cm diameter and bed height of 85 cm. The bed volume of approximately 135 cm^3 was calibrated by passing elution buffer at a flow rate of 0.3 ml/min thus 18 ml/h by gravity for 12 h. In order to standardize and run the column, the void volume was estimated to be 43.5 cm^3 by determining the retention time for blue dextran-2000. The column was eluted with phosphate buffer, pH 7.6 collecting 3 ml fractions using a Bio-Rad fraction collector. The elution of the proteins was monitored by reading the absorbance at 280 nm using DU 530 spectrophotometer. The peak fractions were used for activity assay and fractions with CPS I activity were pooled and concentrated using polyethylene glycol-8000, dialysed against several changes of buffer and stored at -20°C . Comparative sephadex G-150 column was prepared as in sephadex G-200 the column loaded and eluted in phosphate buffer, pH 7.6 while collecting 0.3 ml fractions

Native polyacrylamide gel electrophoresis

The method used to purify CPS I protein further was based on the 10% native PAGE gel system first described by Davis (1964). A volume of 20 μl of; the crude CPS I, 35, 50 and 80% ammonium sulphate fractions, and column chromatography peak fraction samples were mixed with equal volumes of sample buffer (0.125 M Tris/HCl pH 6.8, 10% glycerol and 0.002% bromophenol blue) and loaded onto a 12% native-PAGE gel. A volume of 10 μl molecular weight standards (Sigma) were run alongside the samples. The gels were run at a constant voltage (70 V) in 25 mM Tris-glycine buffer, pH 8.3 until the bromophenol blue tracking dye reached the end of the gel. The gels were then silver stained as described by Wray et al. (1981). Colour development was achieved by immersing the gels in a colour developer consisting of 2.5 ml of 1% citric acid (w/v) and 125 μl of 38% formalin adjusted to 250 ml with distilled water until a clear coloration was achieved. This reaction was stopped using a 5% acetic acid and the gels stored in 7% acetic acid prior to photographing.

Protein determination

A protein standard curve was constructed using bovine

serum albumin (BSA) fractions diluted with water to give concentrations ranging from 0 to 0.3 mg/ml. Protein content of liver homogenates and mitochondrial preparations were measured by the procedure of Lowry et al. (1951). Protein concentrations of the samples were calculated from the known concentration of BSA extrapolated from the standard curve.

Coupled assay for CPS I

CPS I was assayed by coupling ADP formation to NAD^+ formation with PK and LDH (Elliot and Tipton, 1974; Corvi et al., 2001) as illustrated in Figure 1. The reagents used were as described by Cheung and Rajiman (1980). The assay mixture consisted of 50 mM Tris HCl buffer, pH 7.4; 15 mM KCl; 5 mM MgCl_2 ; 50 mM NH_4Cl ; 50 mM KHCO_3 ; 5 mM NAG; 10 mM ATP; 45 mM PEP; 5 mM NADH; PK and LDH diluted in 50 mM Tris HCl buffer to a final concentration of 1300-1400 units/ml. The assay system contained isolated CPS I. The assay mixture was pre-incubated at 30°C for 5 minutes in a water bath to allow the temperature to equilibrate and any endogenous pyruvate and ADP to be consumed. The reaction was started by addition of 10 mM MgATP to give a final volume of 3.0 ml. Formation of NAD^+ was followed spectrophotometrically by the decrease in absorbance at 340 nm. The allosteric effect of NAG and NCLG on CPS I activation at various concentrations of the activator was determined in the same method.

RESULTS

Effect of diet on rat sacrificial weight

The mean ($\pm\text{SE}$) initial and sacrificial weight of rats fed on normal and high protein diet were compared (Figure 2). Normal diet rats group, gained mean weight from 306.8 ± 29.2 to 326.5 ± 34.0 g in 10 days at mean rate of 6.4% while rats fed on high protein diet lost mean weight from 269.5 ± 15.5 to 218.8 ± 2.5 g at mean rate of 18.6%. The mean percentage gain in body weight for the control group was lower compared to the net mean percentage weight loss of the two high protein diet groups. Similarly the mean weight gained or lost during at sacrifice was positively correlated to the total mean initial weight of the rat before and after feeding with the two diets. Mean liver weights of the control group at sacrifice was 9.2 ± 1.6 g and of rat fed on high protein diet was 6.5 ± 1.0 g. The weight of the liver tissue was directly and positively correlated to the total body weight of the rats.

Isolation of the mitochondria

Differential centrifugation of the liver tissue at various

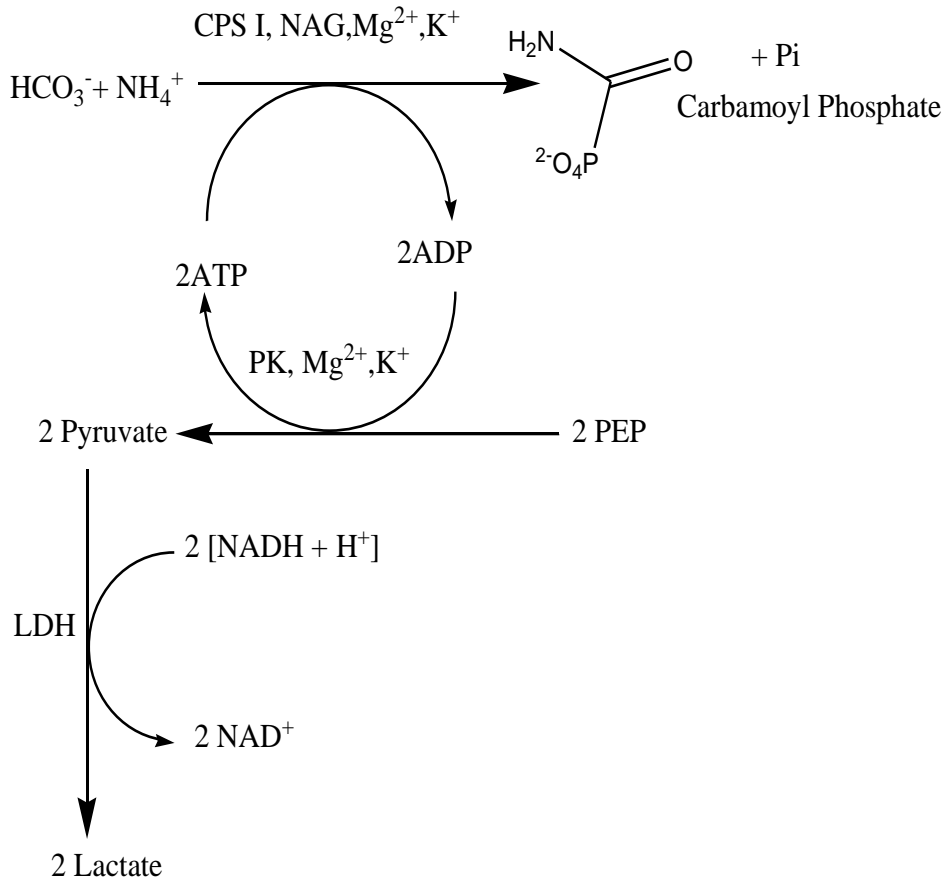


Figure 1. Illustration of the devised coupled assay system for measuring CPS I activity involving lactate dehydrogenase and pyruvate kinase.

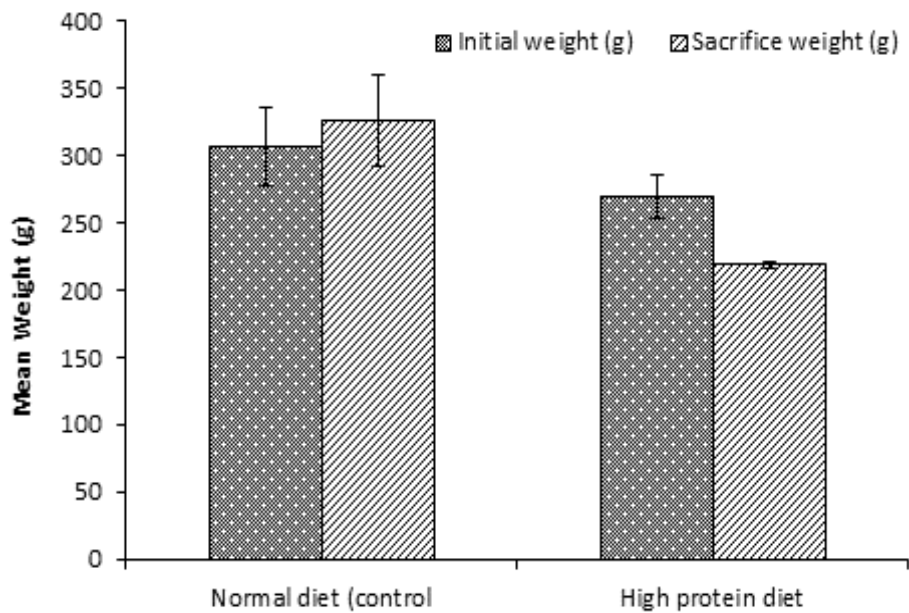


Figure 2. The mean (\pm SE) initial and sacrificial total body weight variation in normal diet (control) and high protein diet rat groups.

Table 1. Fractionation of rat liver homogenate by differential centrifugation.

Differential centrifugation fraction	Centrifuge speed (rpm) and time	Protein concentration (mg/ml)	Total protein yield (mg/g)	Glutamate dehydrogenase (GDH) assay	
				Specific activity (nmol/min/mg)	Total activity (nmol/min/g)
Cytoplasmic extract	2,000 -10 min	45	54	10.43	563.22
Heavy mitochondria	5,500 -10 min	35	41.6	1.11	46.09
Light mitochondria	12,000 -15 min	17	20.4	44.44	864.96
Microsomes	18,000 -30 min	25	30.12	4.66	140.45

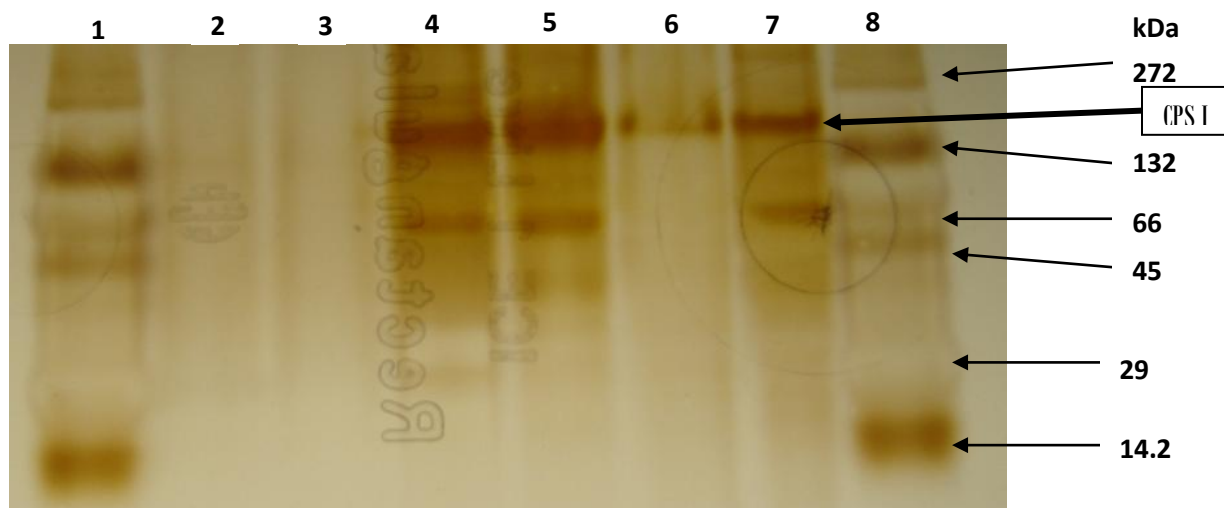


Figure 3. A 10 % native PAGE analysis of the mitochondrial matrix fractions. Lane 1 and 8: Molecular weight markers (range of 14.2-272 kDa), 7: Mitochondrial matrix, 6: 35 % ammonium sulphate fraction 5: 50 % ammonium sulphate fraction, 4: 80% ammonium sulphate fraction, 3 and 2: Sephadex G-200 major peak fractions.

speeds yielded five fractions shown in Table 1. The 12,000 rpm fraction gave the highest value of GDH total velocity of 864.96 nmol/min/g followed by cytoplasmic extract with GDH activity of 563.22 nmol/min/g. Cytoplasmic extract contained all organelles including the mitochondria. For the subsequent mitochondrial isolations the homogenates were centrifuged twice at 2,000 rpm and then at 12,000 rpm and this was able to pellet all mitochondria in one fraction after 30 min. The presence of GDH activity in the microsomal fraction was as a result of simple pouring probably leading mixing of the loosely packed mitochondria after centrifugation at 12,000 rpm. The yield of the mitochondria was in the range of 15-20 mg/g of the wet weight of rat liver.

CPS I partial purification

CPS I partial purification was achieved

The ammonium sulphate purification step of the supernatant of 1% Triton X-100 centrifugation step

yielded three fractions with protein concentration ranging from 22.8-36.9 mg/ml. Further purification was achieved when 3 ml of the 80% ammonium sulphate fraction was run on sephadex G-200 and comparative sephadex G-150 gel filtration. The Separation occurred dependent on protein retention time in the gel. Sephadex G-200 showed two well resolved peaks one major peak at column elution volume (V_e) of 60 ml and a smaller peak at V_e of 185 ml. Sephadex G-150 showed two peaks one well resolved at V_e of 20 ml and a broad peak at V_e of 185 ml. The major peak appeared immediately after the void volume (V_0) in the sephadex G-150 column compared to sephadex G-200 column thereby giving a higher retention time of about 3 hours at the flow rate of 18 ml/h and better separation. 10% native PAGE of mitochondrial matrix protein, ammonium sulphate fractions and sephadex G-200 major peak elution fractions resulted in better separation of CPS I (Figure 3). Lane 4, 5 and 7 shows a prominent band at the region of 132-272 kDa. This region is the region of CPS I as previously demonstrated by Lusty (1978). No bands are seen on Well 4, 6 and 7 probably due to low

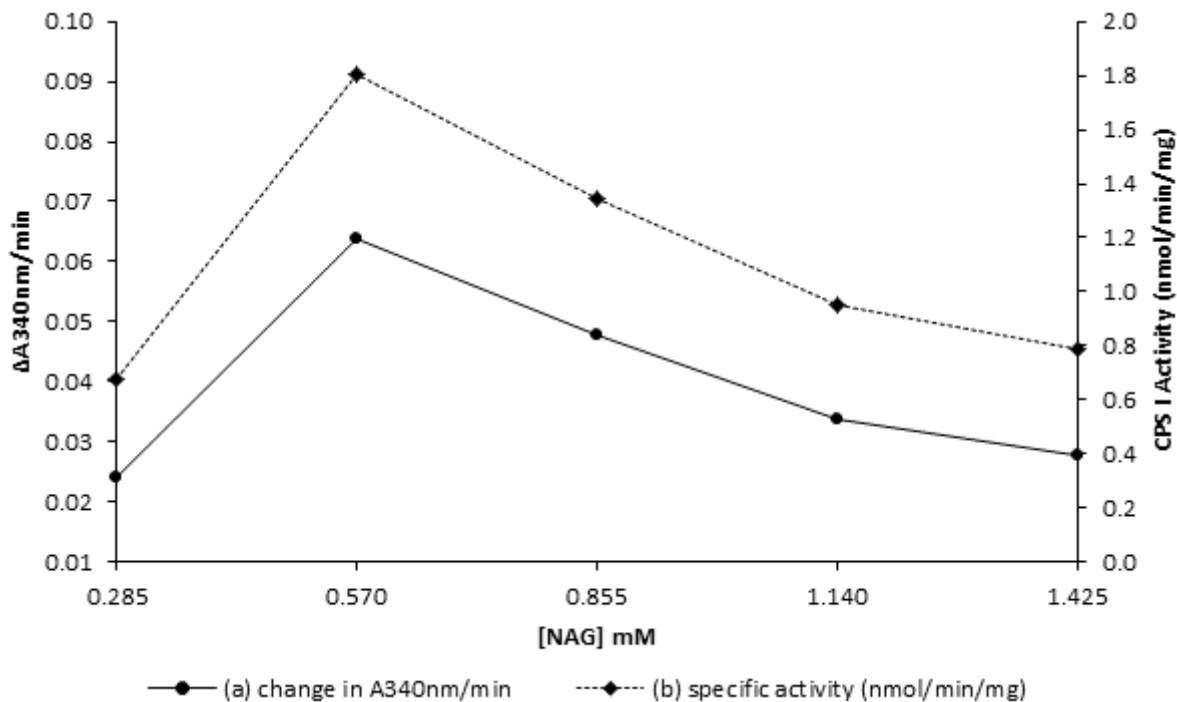


Figure 4. Activation of CPS I by NAG for the rats fed on normal laboratory diet. The activity of CPS I was expressed as (a) the change in Absorbance (A) 340 nm/min or as (b) specific activity in nmol/min/mg protein.

concentration of proteins in those fractions or absence of CPS I.

Coupled assay for CPS I

Activation of CPS I by NAG

The assay devised for the determination of rat liver mitochondrial CPS I activity employed ADP formation coupled to NAD^+ formation via PK and LDH and the decrease in absorbance at 340 nm was determined, excess of PEP and PK were always present to ensure regeneration of ATP. It was observed that for well constituted NADH, the initial absorbance reading at 340 nm was above 1 unit. All the assays for CPS I were performed for 15 min. The effect of the concentration of NAG on modulation of CPS I was studied (Figure 4). It was observed that activity of CPS I at suboptimal concentration of ATP, ammonia and bicarbonate was determined by the amount of NAG present. This enzyme preparation showed 0.57 mM NAG gave the optimum activity in activation of CPS I. Higher values were inhibitory as shown in lowered specific activity of CPS I.

Effect of high protein diet on activity of CPS I

The effect of diet on the activity of CPS I was investigated

in the coupled assay for CPS I (Figure 5). The assay for CPS I showed higher initial velocity in rats fed on high protein diet compared to the rats fed on normal diet (control group). CPS I activity for the 80% ammonium sulphate fraction showed that activity of rats fed on normal diet was 0.042 $\mu\text{mol}/\text{min}/\text{mg}$ while the activity almost doubled to 0.071 $\mu\text{mol}/\text{min}/\text{mg}$ in rats fed on high protein diet..

Comparative activation of CPS I by NAG and NCLG

The effect of NCLG structural analogue for NAG on CPS I activity was investigated. Comparative CPS I assay (Figure 6) for rats fed on normal laboratory diet gave initial velocity of CPS I of 3.07 nmol/min/mg when activated by 0.57 mM NAG and a lowered activity of 2.2 nmol/min/mg when NAG was replaced with 0.57 mM NCLG at suboptimal requirements for other substrates. CPS I activity was higher in NAG modulated than NCLG by 0.87 nmol/min/mg. The activity of CPS I at varying concentrations of NAG and NCLG was also investigated (Figure 7). It was observed that all concentrations of the two activators tested, the enzyme showed higher activity when modulated by NAG compared to NCLG.

DISCUSSION

The effect of diet on the rat means sacrificial and liver

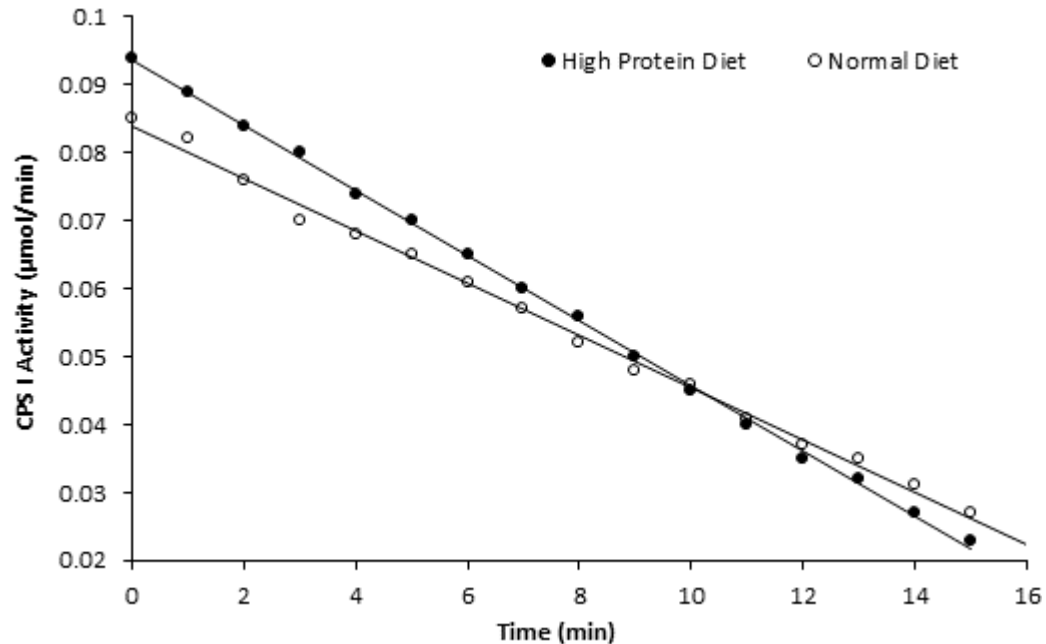


Figure 5. CPS I time-activity plot for normal diet (control) and high protein diet rats. Rats fed on high protein diet showed increased activity compared to rats fed on normal diet at 0.57 mM NAG.

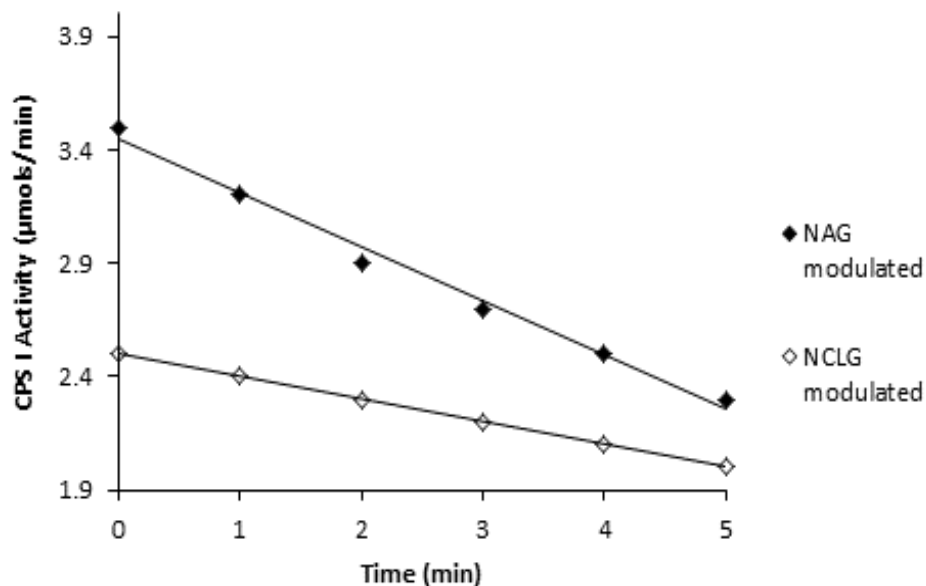


Figure 6. Comparative CPS I modulation by NAG and NCLG with the initial velocity measured for 5 min.

weights were investigated. The results showed the weight of the liver tissue to be directly proportional to the sacrificial body weight. The rats fed on high protein diet had a lower mean sacrificial and liver weight than the control group. Previously, rats fed on high protein diet

was shown to have a lower body weight than normal diet and this was associated with a lower adipose tissue mass and a reduced proportion of large adipocytes (Blouet et al., 2006). Rats fed on high protein diet showed increased CPS I activity compared to normal diet rats.

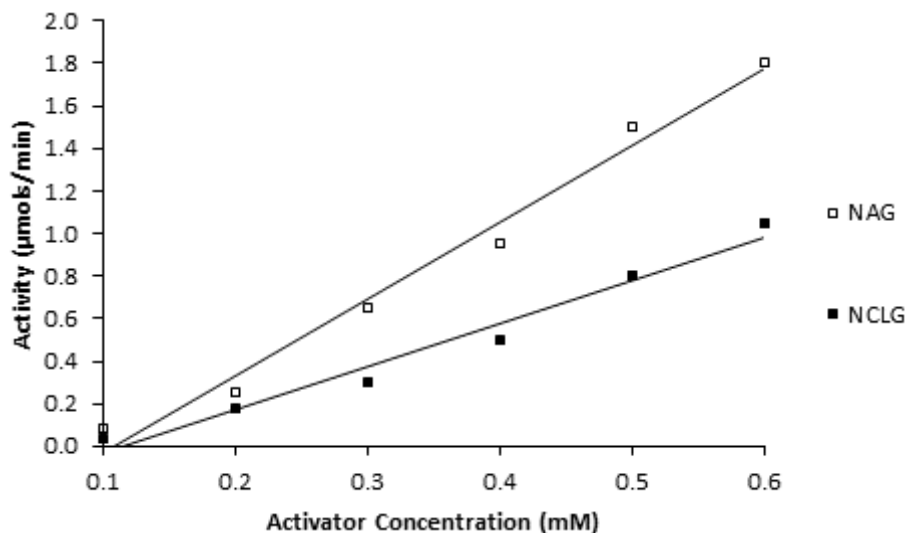


Figure 7. CPS I activity at different concentrations of activator NAG and its structural analogue NCLG.

Previously, it was explained that in well fed state, amino acids originating from digestion of dietary proteins in the gastrointestinal tract were absorbed into the bloodstream and is used for protein synthesis while surplus amino acids especially in high protein diet is metabolized to glucose and then used for fatty acid synthesis or catabolised to generate energy (Morris Jr., 1992). The amino group of amino acids is removed by transamination and deamination prior to urea synthesis in periportal hepatocytes inducing CPS I, although the residual carbon skeleton was suggested to be metabolized to gluconeogenic precursors (Ryall et al., 1984). In starvation, proteolysis of muscle protein was suggested to be the main source of circulating amino acids (Corvi et al., 2001). Morris Jr. (1992) reported that levels of urea cycle enzymes were co-ordinately induced by conditions involving increased protein and amino acid catabolism, such as increased dietary intake and starvation. Similarly, Snodgrass and Lin, (1981) reported that activities of urea cycle enzymes are highest in response to starvation and high protein diets and reduced in response to low protein and protein free diets. Diet dependent changes in urea enzyme activity was primarily as resultant of changes in enzyme mass (Tsuda et al., 1979) suggesting an altered enzyme synthesis rates that correlated with increased activities of these enzymes (Ryall et al., 1984).

Partial purification of rat liver mitochondrial CPS I involved in carbamoyl phosphate synthesis in the urea cycle was achieved in the study. The mitochondria were isolated in presence of 0.25 M sucrose at neutral pH and gave an impressive yield of 15-20 mg/g of the liver. Previously similar yield of 20 mg/g was reported (Myers and Slater 1957). High levels of glutamate

dehydrogenase activity was reported in mammalian liver (Spanaki and Plestakis, 2012) hence a good marker enzyme for the mitochondrial fraction. Previous studies indicated that mitochondria isolated in the presence of sucrose gave the best enzyme activity (Lusty, 1978). Lysing the mitochondria with 1% Triton X-100 did not inhibit CPS I activity. This was in agreement with the previous suggestion that CPS I activity measured as the rate of carbamoyl phosphate formation, appeared maximal when 0.02% Triton X-100 was added to the assay mixture and that higher concentrations of up to 1% were observed to be non-inhibitory to CPS I activity (Lusty, 1978). Clarke (1976) reported that in presence of Triton X-100 the CPS I mainly exist in monomeric and catalytically active form. CPS I was suggested to be stable in preparations of intact mitochondria isolated in sucrose at pH 7 (Lusty, 1978) and that the pH optimum of the purified enzyme was pH 6.8-7. Sephadex G-200 gel filtration of the 80% ammonium sulphate fraction gave a better separation compared to sephadex G-150 column. It was previously observed that sephadex G-200 gave better separation for proteins of molecular weight in the range of 40 kDa to 200 kDa or in better swollen gels, it was observed to extend to 500 kDa (Andrews, 1965). The useful working range of sephadex G-200 suggested to depend on the extent to which the gel was swollen and the exclusion range of this gel was in the limit to separate CPS I with an estimated molecular weight of 160 kDa (Corvi et al., 2001).

Coupling three enzymes in an assay system was demonstrated in the LDH-PK coupled assay devised to measure CPS I activity. The activity was measured as decrease in absorbance at 340 nm corresponding to the formation of one mole of carbamoyl phosphate from one

mole of ammonia and one mole of bicarbonate while utilizing two high energy bonds of ATP. The activity was expressed as the amount of carbamoyl phosphate produced assuming a stoichiometry of carbamoyl phosphate produced per 2 moles of ADP released as was described by Corvi et al. (2001). The enzyme was shown to be activated by one molecule of NAG while one molecule of freshly prepared NADH was oxidized to NAD⁺ as suggested by Stewart and Walser (1980) who hitherto observed that a moderately rapid adaptation of CPS I activity, required 20-30 minutes mediated by NAG the obligatory allosteric activator of the enzyme. Activation of CPS I by NAG and its structural analogue NCLG was evaluated. NAG was shown to be a better activator of CPS I than NCLG at all concentrations assayed. Previously, NCLG has been shown to activate CPS I though with lowered affinity (Caldovic and Tuchman, 2003). Despite CPS I low affinity for NCLG this activator has been used in treatment of patients suffering from hyperammonemia due to NAGS deficiency (Daniotti et al., 2011) based on the suggestion that NCLG could activate CPS I by penetrating the mitochondria and stimulating ureagenesis (Tuchman et al., 2008). Despite being a better activator of CPS I NAG is not been used in treatment of hyperammonemia as it was observed to be degraded in the cytosol by enzyme amino acylase, while NCLG was observed to be resistant to intracellular degradation (Häberle, 2011). Furthermore, studies in rats showed that NCLG could activate the hepatic CPS I and this conclusion was drawn on the observation of decrease of ammonia and an increase of urea concentrations in the blood respectively, as well as consequent increase in CPS I activity in the liver (Bachmann et al., 1982).

Conclusion

LDH-PK coupled assay system was devised to measure CPS I activity in partially purified enzyme preparation by following formation of NAD⁺ by decrease in absorbance at 340 nm wavelength. High protein diet was found to enhance CPS I activity, Allosteric activator NAG and its structural analogue NCLG activated CPS I in the coupled assay system with NAG being a better activator than NCLG.

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