Glucose Tolerance Factor Activity of Binary Amino acid Chromium Complexes in Animals and yeast

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Abstract

This study has evaluated the role of ammo acid - chromium complexation in Glucose Tolerance factor (GTF from yeast has long been known to have trivalent chromium presumably complexed to several amino acid residues but structural details have remained elusive. In this investigation, AA.Cr (AA=amino acid, AA:Cr^{3+} =1:1) synthesized with AA being glutamine, cysteine, glycine, - the putative ligands in GTF-, as also phenylalanine and lysine have been individually examined for their abilities to enhance rates of glucose metabolism in yeast as well as for their GTF activity in the Oral Glucose Tolerance test (OGTT) in rats. For the first time, seminal findings have been made which established that some AA.Cr exhibit powerful and intrinsic GTF activity in both experimental systems with Gln.Cr > Cys.Cr> Gly.Cr>Phe.Cr. This revealed that binary complexes themselves possess necessary and sufficient structure(s) that endow them with GTF activity. Since Phe.Cr was also active, the amino acids presumed to be important in GTF, are not necessarily the only ones with this property. Furthermore, Lys.Cr was found herein to be totally inactive as GTF suggesting that several, but not a with AA.Cr have been discussed, in the context of the role of chromium in cellular metabolism

1. Introduction

The classical studies of Schwartz & Mertz (1959) lead to the isolation of the Glucose Tolerance factor (GTF) from Brewer's Yeast and its identification as a nutritional factor containing trivalent chromium. GTF was found to have a specific role in regulating glucose metabolism in animals. On the assumption that in GTF, trivalent chromium is complexed to glutamic acid, glycine, cysteine and also nicotinic acid, Toepfer et al (1977) prepared synthetic "GTF" by refluxing chromium acetate with a mixture of these amino acids and nicotinic acid. They claimed that the product obtained was very similar to the yeast isolate in physicochemical as well as biological properties. However, in neither case is the nature and content of chromium complex(es) established.

GTF activity is characteristically quantitated, in experimental animals, by the Oral Glucose Tolerance Test (OGTT). Also since it was shown that exogenously provided GTF, but not free Cr^{3+}, enhanced the rate of glucose metabolism in yeast, attempts were made to develop yeast assays for GTF with *S.carlbergenesis* (Holdsworth & Appleby, 1983) or *S. ellipsoideus* (Haylock et al, 1983). Such assays have also been utilized to quantify GTF especially during fractionation and purification of GTF isolates (Haylock et al, 1985, Zetic et al 2001). However as was pointed out by Zetic "nobody has isolated pure GTF todate" and the exact structure(s) of GTF remains an enigma todate.

The search for different bioactive forms of chromium led to the discovery of certain naturally occurring oligopeptides with GTF activity. LMWCr (Low Molecular Weight Chromium Binding Substance), one of the first to be isolated by Davis & Vincent (1997) presumably has four Cr^{3+}, in a multinuclear assembly, bound to amino acid residues of the oligopeptide ; however, LMWCr has aspartic acid, in place of glutamic acid in GTF and also lacks nicotinic acid. It belongs to a class of similar oligopeptides that occur in different animal tissues (Yamamoto et al, 1987; 1988).

More recently, Yang et al (2005) synthesized $(D\text{-}Phe)_3.Cr$ and showed that it improves glucose tolerance and also signaling at the insulin receptor level. Their findings constitute the first

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demonstration of a complex of defined structure with GTF like activity. However, $(D.Phe)_3.Cr$ is a ternary complex of an unnatural D-isomer that is moreover, not a presumed ligand for Cr^{3+} in either GTF or LMWCr.

The present work stems from the premise that binary amino acid-chromium complexes wherein AA:Cr^{3+} =1:1 are in fact, the legitimate model compounds to address the question of the structural requirements for GTF activity. Also our recent work indicated that in AA.Cr synthesized under conditions analogous to those that prevail in yeast, when it elaborates GTF (Karthikeyan et al, 2010 in press), the metabolic function of the amino acid complexed is profoundly modified as shown by the properties of Lys.Cr (Karthikeyan et al., 2008). During these studies, preliminary observations had been made that some AA.Cr also stimulated glucose metabolism in yeast (Karthikeyan et al, 2010, loc cit). Consequently binary chromium complexes of glutamine, cysteine, glycine and lysine have been synthesized and examined in *S. cerevisiae* (NCIM 3559), for their ability to enhance the rate of glucose utilization ; corresponding OGTT was also conducted in rats to evaluate whether the activities in the two test systems were concordant, thereby reflecting classical GTF activities. The outcome of such experiments has lead to the seminal finding that binary AA.Cr are, in fact, GTFs and that higher order structure is not mandatory. It has also emerged that the procedure devised herein for assaying GTF activity with *S.cerevisiae* (NCIM 3559) is much simpler than procedures currently available with other yeast strains and can therefore be helpful in screening for GTF.

On the basis of the generality of the effects of AA.Cr and the nature and magnitude of the specificities observed, insights gained regarding the enigma of GTF structure and, in general, on the importance of amino acid chromium interaction in the biological role of chromium in cellular metabolism have been highlighted.

2. Materials & Methods

2.1. Synthesis of Amino acid Chromium Complexes

Binary AA.Cr (AA:Cr^{3+} = 1:1) were synthesized by reacting equimolar amounts of amino acid and trivalent chromium (as chromium sulfate) at 45±1^{0}C and a pH of 4.0± 0.1. These conditions have been designed to simulate those that prevail during elaboration of GTF by yeast. The AA.Cr obtained are stable and water soluble and their structures have also been deduced from their IR spectra (Karthikeyan et al, 2008, in press 2010). Generally, the carboxyl of the amino acid is bound firmly to Cr^{3+} in unidentate fashion, as indicated by the strong IR frequency of v_{as} (COO^-), with additional contribution from amino-N coordination. The complexes are formed from trivalent hexaquo chromium, from which the bound water molecules are displaced by the carboxyl and amino groups to yield octahedral complexes. (KS Karthikeyan et al, 2010 in press)

2.2. Maintenance of Yeast (S.cerevisiae NCIM 3559) cultures

Saccharomyces cerevisiae (NCIM 3559) was routinely maintained on slants containing Yeast Extract (1%), Glucose (2%) , Peptone (2%) & Agar (2%) by sub culturing twice a week.

For metabolic studies, *S.cerevisiae* (NCIM 3559) was maintained on CBM, Watson, 1983) with 1% glucose. From preliminary tests, the minimal optimal requirement for enhancement of glucose utilization rates was found to be 1 mM AA.Cr. Lys. Cr, Phe. Cr. Gly.Cr, Cys. Cr and Gin. Cr were tested at this concentration in experiments to determine their influence on the rate of glucose metabolism.

2.3. Studies on Initial kinetics of Glucose Utilization by S.cerevisiae (NCIM 3559)

For studying kinetics of glucose utilization, yeast cells (*S.cerevisiae* NCIM 3559) were grown at 30 ± 1^{0}C for 24 hrs. to log phase (Optical density at 660 nm of 1.5) and cells centrifuged at 1^{0}C at 10,000 rpm for 10 min, in an Eppendorf 1510 R ultracentrifuge ; the supernatant was discarded and the cell pellet was transferred under sterile conditions to a (CBM-Glucose) medium and incubated for 30 minutes at 30 + 1^{0}C. After this, cells were collected by centrifugation and were thereafter transferred to fresh CBM containing ImM AA.Cr, shaken to disperse and allowed to stand cell concentration has routinely round 10^8 cell per it this stope.. At intervals of 2 hrs, over the next ten hours, aliquots of the cell suspension were withdrawn, subjected to centrifugation (as before) and residual glucose in the supernatant was estimated by a standard DNS (Dinitrosalicylic acid) method (Miller, 1972).

After decanting the supernatant, the cells were lysed with TCA (10%) and protein was extracted with NaOH (IN) ; following pelleting out of debris by low speed centrifugation the alkali supernatant was used to quantitate protein by a standard biuret procedure (Layne, 1957)

Cell counts were performed using modified Ringer's solution (Thomas and Ingledew 1992) in a Neubach chamber. The rate of glucose utilization was expressed as)imoles/mg protein / min.

2.4. Oral Glucose Tolerance Tests In Rats

Rats belonging to the Sprague - Dawley strain , 3 in number (1 year old) were used for OGTT (Oral Glucose Tolerance Tests). The rats were fasted for 18 h. prior to OGTT and 25% Glucose (Iml/lOOg body at) was administered orally. Complexes were then administered orally at a concentration Of 45 µg / kg body wt (Dong et al., 2007)

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At intervals thereafter blood was drawn (~ 2ml) from the supra-orbital sinus of the rats and glucose levels in the plasma were estimated by a glucose oxidase peroxidase method using a standard kit. Fasting glucose was also analysed & recorded.

Blood glucose levels were graphically plotted against time on graph sheets (cm. graphs) and curves were drawn connecting the points. The Area Under the Curve (AUC) was obtained by manual counting of squares for quantization of OGTT. The lower the AUC, the more effective is the AA.Cr. All experiments were repeated at least thrice, with three rats per group. All the animal studies were approved by the animal ethical committee National Institute of Nutrition Hyderabad (India).

3. Results and Discussion

Figure 1 displays the effects of exogenously provided AA.Cr (ImM)on glucose utilization by washed and briefly starved log phase cells of *Saccharomyces cerevisiae* (NCIM 3559). Since linear rates prevailed upto the first ten hours, as evident from the graphical plots, the kinetics of glucose metabolism could be quantitated from these data. Several of the AA.Cr examined herein are those that would be expected to be contributory to the activity of isolated yeast GTF. However, Gin. Cr has been used in place of Glutamate-Cr since the latter was found to be unstable. Phe.Cr has also been tested in view of the reported activity of $(D\text{-}Phe)_3$ Cr (loc cit) and is also, not unexpectedly, active. Furthermore, it is evident that magnitude wise, the order of activity is Gln.Cr > Cys.Cr> gly.Cr> Phe.Cr. This implies that chromium complexes of all putative amino acid ligands of yeast GTF exert a profound influence on glucose metabolism in yeast. Quite possibly, they are among the more active, as compared with complexes of other amino acids such as those of phenylalanine and lysine. Lys.Cr does not have any effect on glucose metabolism in yeast, suggesting that several, but not all AA.Cr possess GTF activity. This is interesting for another reason as well. Yeast is unable to utilize free lysine as a sole nitrogen source. However, it was shown recently (Karthikeyan et al 2008 and 2010, in press') that Lys. Cr, on the other hand, is a good sole N source. Thus chromium complexation herein, profoundly influences metabolic properties of the amino-N of this amino acid, but does not endow the complex with GTF activity. Evidently, the nature of the effects of chromium complexation is amino acid specific.

However, the critical test is whether the remarkable effects of AA. Cr in yeast translate into equivalent GTF activity in experimental animals. This was therefore, next examined, with AA.Cr being orally administered at levels (of 45 µg/ Kg. body wt.) corresponding to those employed by Dong et al (2007 loc. cit). The results obtained are graphically plotted and shown in Figure 2.

It is clear that all AA.Cr effective in yeast are also comparably active in the OGTT. Furthermore, as in yeast, Gin. Cr > Cys. Cr > Gly. Cr > Phe. Cr with Lys.Cr totally lacking in ability to facilitate the disposal rate of a glucose load. AA. Cr of the amino acids of GTF themselves therefore qualify as "GTFs" as attested to by the positive as well as negative outcomes in both tests found herein as well as from the magnitudes of the responses calculated and tabulated in Table 1.

It may be seen that the group of AA.Cr - of Gin, Cys. Gly, - enhance the rate of glucose metabolism to levels of ~ 200% or higher over the control. Although comparisons are rather difficult to make, in view of the uncertainties regarding structural features of natural GTF, it is interesting that Holdsworth and Appleby (1984) had also reported a > 2 - fold increase in their yeast assay for exogenously added natural "GTF".

One interesting feature of the yeast assay procedure devised herein is that it is not only definitive for GTF, but is also much

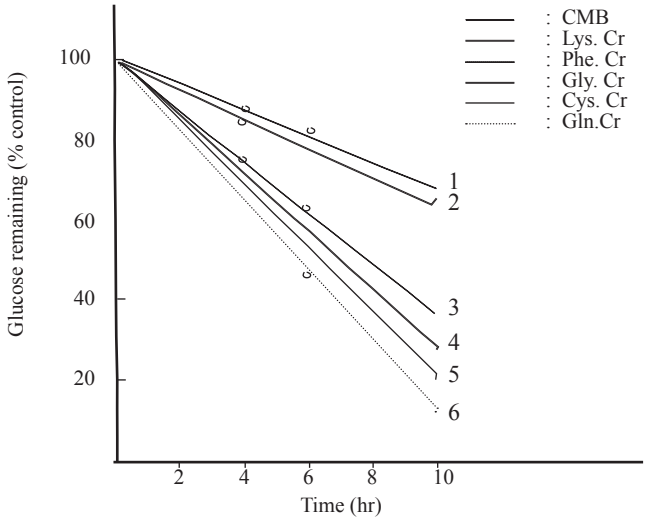

Figure 1: 1Effects of AA.Cr (ImM) on initial rates of utilization of glucose (µmole / min / mg protein)

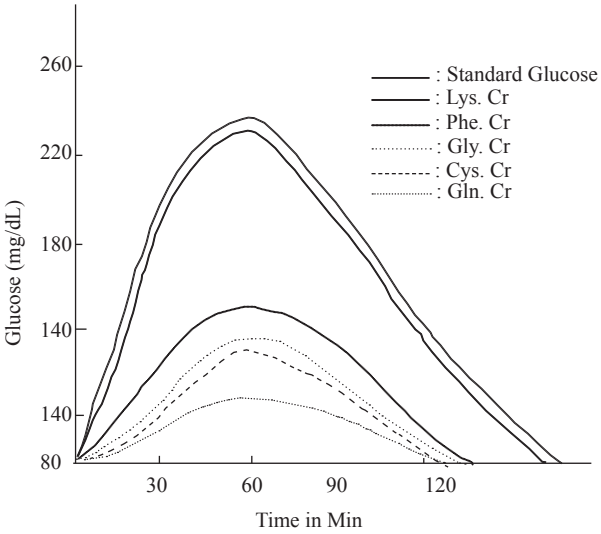

Figure 2: Effects of AA.Cr (45 µg / kg body wt) on OGTT in rats

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Table 1 : Quantitative effects of AA.Cr as GTF in animals & yeast

AA.Cr -	Glucose metabolism (yeast) Rate*	% control	OGTT (Rats) Area under Curve (+)	% control
Control (None)	0.10(0.08)	100	47.87 (4.3)	100
LysCr	0.11 (0.08)	110	45.90 (2.9)	96
Phe.Cr	0.19(0.09)	190	19.54(1.7)	41
Gly.Cr	0.22 (0.09)	220	13.61 (2.2)	28
CysCr	0.24 (0.09)	240	11.81 (1.3)	25
Gin. Cr	0.27 (0.09)	270	6.98 (0.08)	15

- Ar 1 mM in yeast; 45 kg / body wt in rats * μ moles glucose / mg protein / min + Area counted in Sq. cm from graphical plots Values in brackets : +S.D. Experimental details, see text

simpler than existing methods (e.g. 'Holdsworth & Appleby, 1984). The other procedures demand that the yeast cells be first made chromium deficient and then starved for glucose. Such assays are based on increase in $^{14}CO_2$ generated from radio labeled glucose under the influence of GTF. However, part of the $^{14}CO_2$ produced also gets concomitantly fixed into cell material due to the pyruvic carboxylase of the cells. Conceivably, this could be a complicating factor when assaying GTF from different sources. On the other hand, this strain does not have such mandatory requirements. The assay, based on large magnitude changes in glucose metabolism is more straightforward. It should prove useful in probing structure - activity correlations that determine GTF activity.

Importantly the results of the present investigation make it possible to address the enigma of the structure of GTF elaborated by yeast. Isolation of GTF from yeast as well as synthesis, from amino acids and trivalent chromium of similar material as reported by Toepfer et al (1977), involves certain common chemical steps. These are, briefly, initial treatment with strong acid (refluxing with 5 N.HC1) followed by adsorption onto a resin matrix and eventually elution with ammonia (IN. NH_4OH) from the ion exchange resin, Significantly, these are also very similar to procedures first laid down by Ley and Ficken

(1912) for synthesis of AA_n. Cr where n=2 or 3, the complexes, formed are often insoluble and their detailed structures are not defined.

In this context, the chemistry of trivalent chromium assumes importance in aqueous solution , two different kinds of reactive chromium species occur : hexaaquo chromium below pH 6.0 and olated, multimeric species with several Cr^{3+} linked by hydroxyl bridges, at pH > 7.0 ; reaction of amino acids with these reactive species leads to different kinds of amino acid complexes whose structures and biological properties are not fully known (De Pamphilis and Cleland, 19731 consequently, treatment of GTF could also modify the resident, native, amino acid complexes and until the nature, extent and contributions of the modified forms is known, it is evidently difficult to be certain about the structures that prevail. However, the unequivocal demonstration, herein, of intrinsic GTF activity resident in binary complexes emphasizes that such complexes themselves are also very likely the truly active structures in GTF in vivo.

4. Conclusion

Binary AA.Cr have in themselves, necessary and sufficient structure(s) that endow them with GTF activity are seminal findings which constitute the first insights into the structure activity correlations that are central to the biological role of complexed chromium in cellular metabolism.

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