Metabolism and Glucose Tolerance Factor Activity of Synthetic Amino acid-Chromium Complexes in Yeast

K.S. Karthikeyan¹, P. Nagababu², K. Sivarama Sastry² and S. Satyanarayana²*

¹Endocrinology and Metabolism Division, National Institute of Nutrition, Jama-i-Osmania P.O. Hyderabad – 500007, India
²Dept of Chemistry, Osmania University, Hyderabad-500 007, India
*For Correspondence - ssnsarasani@gmail.com

Abstract
The Glucose Tolerance Factor (GTF), a chromium containing complex, has been isolated from yeast by Schwartz & Mertz. This was instrumental in the recognition of the role of trivalent chromium, complexed to amino acids, in regulation of glucose metabolism and of active GTF as a potential antidiabetic agent. We report herein on newly devised simple, synthetic procedures for soluble binary AA-Cr (1:1) complexes of known structure(s) and on their activities in S.cerevisiae. Lys.Cr permits post exponential growth of yeast and prevents apoptosis, unlike free lysine which inhibits post exponential growth and cell viability. Phe.Cr is a better sole carbon source for yeast than the free amino acid and complexation, in this case, enhances the rate of metabolism of its carbon skeleton. In addition, Phe.Cr as well as Gln.Cr, but not Lys.Cr, exhibit considerable GTF-like activity in yeast. The results obtained suggest that soluble AA.Cr are good model compounds for exploring structure activity correlations that determine the role of trivalent chromium in cellular metabolism.

Keywords: GTF (Glucose Tolerance Factor), Amino acid-Chromium complexes (AA.Cr), ligands, Metabolism, Saccharomyces cerevisiae.

Introduction
The Glucose Tolerance Factor (GTF) was isolated from Brewer’s yeast (1) as a water soluble complex containing ~ 5-6% trivalent chromium and glycine, glutamic acid and cysteine as well as nicotinic acid as putative ligands (2). A material claimed to be similar to the isolate in many aspects was also synthesized by Toepfer et al. (2) by refluxing chromium acetate with these amino acids as well as nicotinic acid. However, the exact nature of the complexes remains unknown in both cases. Several researchers like Galuszka et al., (3) have synthesized different AAn.Cr (where n=2 or 3 and AA=Amino acid) by classical organic synthetic procedures, devised by Ley and Ficken in 1912 (4). The products, although non toxic, were poorly soluble, and their biological activity is unknown.

Davis and Vincent (5) isolated, from animal tissues, a GTF-active oligopeptide (Low Molecular weight Chromium Binding material) or LMWCr (6), which contained aspartic acid in place of the glutamic acid in GTF and no nicotinic acid. The first synthetic Amino acid-Chromium complex with GTF-activity (D-Phe)₃.Cr, was prepared by Yang et al (7). However, this poorly soluble complex was rather unusual in that the Amino acid is the D-isomer and phenylalanine has not been considered earlier as a putative ligand in either GTF or LMWCr.

More recently, we (8) had synthesized a soluble Lys.Cr by reacting chromium sulphate with an equimolar amount of L-Lysine under mild,
near physiological conditions akin to those that yeast utilizes to elaborate GTF. This Lys.Cr is a binary(1:1) complex and has been characterized by physicochemical techniques. *S. cerevisiae* was found to metabolize Lys.Cr very differently from the manner in which free lysine was utilized, but Lys.Cr added to metabolizing yeast cells had no effect on their glucose metabolism.

The synthetic procedure used by us for Lys.Cr (8), has now been developed into a rapid, mild and general preparative procedure for binary AA.Cr complexes. Metabolic effects of Gln.Cr, Phe.Cr and Lys-Cr in yeast have been studied. The observed strong GTF like activity of Gln.Cr and Phe.Cr in *S. cerevisiae* is emphasized in view of the utility of such AA.Cr as potential auxiliary therapeutic agents. Structural characteristics of different AA.Cr complexes that determine biological activity of chromium are discussed.

**Material and Methods**

**Synthesis of binary (1:1) Amino acid-Chromium complexes (AA.Cr)**: AA.Cr complexes were synthesized by reacting AA (Phe, Gln, or Lys) with chromium sulfate hexahydrate [Cr$_2$(SO$_4$)$_3$.6H$_2$O]. All chemicals were of AR grade and products of Merck.

Amino acid and Cr$_2$(SO$_4$)$_3$.6H$_2$O, (500 µmoles each in total volume of 6.0 ml) were reacted in a thermostated water bath at 45±1°C, for a period of up to 60 min. at a pH 4.0±0.1. Routinely, complex formation was monitored in a Klett-Type Elico colorimeter with a 57 filter (550-600nm) and designated as “$A_{570}$”.

**Kinetics of AA.Cr formation**: Kinetics of AA.Cr formation was monitored by following increase in $\Delta A_{570} = A_{570}^{AA.O} - A_{570}^{O}$ with time under the reaction conditions. From the graphical plots obtained, $t_{1/2}$ for formation was obtained.

**Spectral features of AA.Cr complexes (UV/Vis,-IR)**: UV/Vis spectra of the AA.Cr complexes were measured in a U-2800 Beckman spectrophotometer over the wavelength range 220-800nm. For IR spectra, recrystallized amino acid-chromium complexes were dried to constant weight over phosphorous pentoxide in a vacuum desiccator. The sample (1.0-1.5mg) was mixed thoroughly with KBr in a (1:100) ratio, pelleted in a KBr press under 6 tonnes pressure and the pellet scanned in a Fourier Transform Thermo Nicollet Nexus 670 Spectrophotometer (resolution 4cm$^{-1}$) over the range of 4000cm$^{-1}$ to 400cm$^{-1}$.

**Thin Layer Chromatography (TLC) of AA.Cr complexes**: TLC plates (15cm x 9cm) were prepared by suspending silica gel (40 g) in sodium carbonate (1mM, 90ml) and spreading the mix on thin layer plates as per standard techniques. The plates were air dried and activated at 110°C for 1h just prior to a chromatographic run. Recrystallized complexes in aqueous solution (50 µg) were spotted in ~ 20-40 µl on to the TLC plates along with amino acid standards as well as Cr$^{3+}$ complexes separately. The solvent system used was butanol: pyridine: water (1:1:1) with running time of ~ 2h. Visualization of AA.Cr and free AA was by ninhydrin spraying.

**Yeast growth experiments**: *Saccharomyces cerevisiae* (NCIM 3558) was routinely maintained on a medium containing yeast extract.
In growth experiments, yeast was grown generally on a complete basal minimal salts medium (CBM) described by Watson (10). This also contained ammonium sulphate (2 mM) and glucose (1%).

For studying post exponential growth (11) for which a suboptimal level of nitrogen in the medium is mandatory, the growth medium contained 0.5 mM NH$_4$+. For studying post exponential growth (11) for which a suboptimal level of nitrogen in the medium is mandatory, the growth medium contained 0.5 mM NH$_4$+. When metabolism of AA-Cr was examined by using them as either Nitrogen N, Carbon C or (N+C) sources for yeast, based on preliminary experiments, each AA-Cr was used at a level of 10 mM in place of NH$_4$+ or glucose or both, respectively in the CBM mentioned above; invariably AA-Cr was separately sterilized in such cases and added aseptically to the rest of the medium which had also been presterilized.

When glucose utilization was to be determined during growth, at the required time, culture flasks were gently shaken so that a uniform cell suspension was achieved, following which aliquots (1.0 ml) were withdrawn and their absorbance (A$_{660}$) measured. Cells were pelleted out by centrifugation at 0°C. From the clear supernatant obtained, glucose present was estimated (12).

**Post exponential growth**: In experiments involving post exponential (PE) growth (11), the following procedures were employed. Yeast was grown for 24 h (20 ml CBM in 100 ml conical flasks) to the exponential phase. Growth was measured and the entire culture was centrifuged under sterile conditions and the supernatant medium was discarded.

The yeast cells were then re-suspended in a known volume of growth medium limiting in nitrogen (0.5 mM NH$_4$+) and transferred to fresh culture flasks containing the rest of the volume, other media constituents (to make to 20 ml) also containing lysine or Lys-Cr as required. Growth was thereafter followed up to 144 h. All operations were performed aseptically, in a laminar flow chamber.

**Results**

**Synthesis of binary (1:1) AA.Cr complexes**: The rather insoluble (AA)$_n$.Cr (n=2 or 3) complexes, made by organic synthetic procedures based on those first employed by Ley and Ficken (4) are quite different from the water soluble, bioactive chromium complexes such as GTF and LMWCr. The procedures developed herein are much milder and designed to be analogous to conditions for binary AA.Cr complex formation first devised for Lys.Cr (8) (These have been examined by varying time, temperature, pH etc., and the conditions finalized herein are the minimal optimal ones). AA.Cr with AA being phenylalanine, lysine, glutamine, aspartic acid, asparagine or glycine have been successfully made accordingly. The data for Phe.Cr, Gly.Cr, and Gln.Cr have been presented herein as typical examples. Fig 1 shows the kinetics of formation of Phe.Cr (Phe:Cr$^{3+}$ = 1:1) and its t$_{1/2}$ is seen to be around 6-7 min. t$_{1/2}$ was found to vary from 6-40 min for different amino acids.

During Phe.Cr formation it was found, for the first time herein, that concomitant with complexation, the aromatic absorption (λ$_{max}$ = 257 nm) of this amino acid was lost leaving only end absorption. Consequently the time course of this loss of absorption has been examined and is shown in Fig. 2. In conformity with the data of Fig 1, t$_{1/2}$ derived from abolition of UV absorbance is also 6 min. The UV/Vis spectrum of Phe.Cr is displayed in Fig 3. The λ$_{max}$ of Phe.Cr is 540 nm.
To determine the stoichiometry of AA.Cr formation, Job’s plot (9) has been modified by keeping the concentration of the chromophoric Cr³⁺ constant and observing increase in 

\[ DA_{570} = \left( A_{570}^{AA\text{Cr}} - A_{570}^{Cr} \right) \]

as a function of AA concentration. When such data are plotted for Phe.Cr (Fig 4), the stoichiometry can be deduced to correspond to that for a binary (1:1) complex. Similar plots for synthesis of other AA.Cr, wherein the amino acid were, for example, glycine, aspartic acid, aspargine or glutamine also confirmed that

---

**Fig. 1.** Kinetics of formation of binary aminoacid chromium (III) complexes.

**Fig. 2** Kinetics of Loss of UV absorbance During formation of Phe.Cr

**Fig. 3.** Absorption Spectrum of Phenylalanine-Cr³⁺ binary Complex (Phe.Cr)

**Fig. 4.** Formation of Lysine-Cr (1:1) Complex (LysCr) : Job’s Plot

**Fig. 5.** IR Spectrum of Phe.Cr (Phenylalanine-Cr³⁺, 1:1 binary complex)

Karthikeyan et al
the procedure devised herein invariably leads to binary complexes.

An examination of the IR spectra revealed the nature of the bonding involved. In Fig 5, 6 and 7 are presented the

IR spectra of Phe.Cr, and Gln.Cr. The relevant $\tilde{\epsilon}_{\text{max}}$ in the visible region and characteristic IR peak frequencies are summarized in Table 1. It can be seen that the $\tilde{\epsilon}_{\text{max}}$(COO) vibrational peak, due to –O-CO-O-Cr is dominant in all complexes. The changes in the $\tilde{\epsilon}$(S=O) vibrational frequencies for chromium sulphate to a single peak at $\tilde{\epsilon}1129$ cm$^{-1}$ for Lys.Cr and Gln.Cr (but not for Phe.Cr) indicate that in some AA.Cr the sulphate is more strongly to bound to Cr$^{3+}$ than in others. The structures derived for AA.Cr are shown in Fig 7.

**GTF activity of AA-Cr in Yeast:** The rate of glucose metabolism in *S. cerevisiae* (NCIM 3558) was first shown to be enhanced by 10mM Gln.Cr

![Fig. 6. IR spectrum of Lysine-Cr$^{3+}$ (1:1) binary complex](image)

![Fig. 7. Possible structures of Amino acid-Cr$^{3+}$ complexes with co-ordination via carboxyl as well as sulfate (R.COOH = AA) (Based on IR Spectra)](image)

I - Hexaaquo - Cr
In II, AA = Phenylalanine
In III & IV, AA = Lysine or Glutamine

![Fig. 8. Phenylalanine-Cr$^{3+}$ utilization by Saccharomyces cerevisiae NCIM 3558.](image)
We therefore examined the minimal concentration of AA.Cr for affecting glucose metabolism in yeast and found that 1mM was adequate. When AA.Cr was also included, along with glucose (1%) in the medium, the time for metabolizing (disappearance) 50% of the glucose was as follows: Control (CBM), 100h; Lys.Cr 91 h; Phe.Cr 50h; and Gln.Cr, 34h. Variations in $t_{50}$ in replicates was no greater than 5-7% of the given values. Thus some binary AA.Cr possess remarkable GTF activity in Yeast.

**Metabolism of AA.Cr in Yeast**

The metabolism of Phe.Cr has also been examined by employing it as a N,C as well as (N+C) source for yeast. As the results in Fig.8 show, phenylalanine as well as Phe.Cr are equally good nitrogen sources. In contrast, phenylalanine is poorly metabolized as a sole “carbon” source and this becomes rate limiting as evident from growth on phenylalanine as a sole (N+C) source. With Phe.Cr, growth is much enhanced over that on phenylalanine alone.

A special feature of the metabolism of lysine in yeast is that lysine strongly inhibits post exponential (P.E.) growth of Yeast. The effect of Lys.Cr on P.E. growth has been studied. Fig. 9 presents the results obtained.

In S. cerevisiae (NCIM 3558), lysine is seen to powerfully inhibit P.E. growth and also lead to an actual decrease in turbidity of the culture, a consequence of cellular apoptosis. In contrast, Lys.Cr permits good P.E. growth and the toxic or Phe.Cr, in our earlier studies (13). We therefore examined the minimal concentration of AA.Cr for affecting glucose metabolism in yeast and found that 1mM was adequate. When AA.Cr was also included, along with glucose (1%) in the medium, the time for metabolizing (disappearance) 50% of the glucose was as follows: Control (CBM), 100h; Lys.Cr 91 h; Phe.Cr 50h; and Gln.Cr, 34h. Variations in $t_{50}$ in replicates was no greater than 5-7% of the given values. Thus some binary AA.Cr possess remarkable GTF activity in Yeast.

**Metabolism of AA.Cr in Yeast**

The metabolism of Phe.Cr has also been examined by employing it as a N,C as well as (N+C) source for yeast. As the results in Fig.8 show, phenylalanine as well as Phe.Cr are equally

---

**Table 1**: Major characteristic vibrational IR frequencies of Chromium complexes and ligands

<table>
<thead>
<tr>
<th>Complex</th>
<th>3500-2000* [uOH &amp; uNH$_2$]</th>
<th>1650-1500 [u$_{\text{IR}}$(Coo)]</th>
<th>1500-1400</th>
<th>1130-1000 [u$_{\text{IR}}$(SO$_2$)]</th>
<th>600 [u$(\text{Cr-O})$]</th>
<th>500-400 [u$_{\text{IR}}$(Cr-N)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine-Cr</td>
<td>3430*; 2929*</td>
<td>1633*</td>
<td>1451*</td>
<td>1136*; 1041*</td>
<td>609*; 498*; 462*</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3449*; 3087*; 2939*</td>
<td>1626*; 1561*</td>
<td></td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lysine-Cr</td>
<td>3424*; 3065*; 2939*</td>
<td>1629*; 1518*</td>
<td>1460*</td>
<td>1129*</td>
<td>609*; 407*</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>2929*</td>
<td>1585*; 1505*</td>
<td>1406*</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Glutamine-Cr</td>
<td>3434*</td>
<td>1669*</td>
<td>1440*</td>
<td>1121*</td>
<td>617*; 453*</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>3409*; 3052*</td>
<td>1672*; 1585*</td>
<td>1406*</td>
<td>-</td>
<td>615*</td>
<td></td>
</tr>
</tbody>
</table>

* Infra red frequencies in Cm$^{-1}$. Regions within which vibrational frequencies of groupings indicated in brackets (†) are found in the complexes examined.

[Strengths of vibrational bands : s-Strong; m-Medium; w-Weak]
effect of the free amino acid is offset by binding to Cr\(^{3+}\).

**Discussion**

The present investigation addresses three major aspects of the role of chromium in cellular metabolism:

(i) Synthesis and structure of water soluble, binary AA.Cr and their importance as model compounds; (ii) the GTF activity of AA.Cr and (iii) the metabolism of AA.Cr in Yeast.

**Synthesis and structure of AA-Cr :** GTF as well as LMWCr are thought to contain Cr\(^{3+}\) bound to amino acids but the structural features of the complexes present therein are unknown. To understand this aspect, it is necessary to synthesize complexes of known structure. In the synthetic procedure of Ley and Ficken (4), which has been generally followed ever since, glycine was reacted with chromium chloride in a 3:1 ratio, for 48h at 60°C. They obtained a red (Gly)\(_3\) Cr. On prolonged boiling in water, this complex lost two amino acid residues to become a binary Gly.Cr. This indicates the greater stability of AA-Cr over (AA)\(_3\) Cr. Over the years, many researchers, likewise, generally reacted trivalent chromium with amino acids and ended up with rather insoluble (AA)\(_n\) Cr (n=2or 3). Under such conditions, the reactive chromium species would be an olated form with the structure or polymers thereof. Such polynuclear forms predominate at pH > 6.0 with multiple linked chromium ions. It has been stressed by Galuszka et al., (3) that the nature of amino acid chromium complexes formed is dependent on the conditions employed and since bioactive chromium complexes are water soluble and stable, a completely different procedure has been developed herein. This is essentially based on the findings of Shuttleworth et al (14), who reacted \(\alpha\), \(\beta\) and \(\gamma\) amino butyric acids with chromium sulphate under mild conditions and showed that AA.Cr type binary complexes do form; such formation was accompanied by a shift of the \(\varepsilon_{\text{max}}\) from that of the Cr\(^{3+}\) as well as an increase in \(\varepsilon_{\text{max}}\) (in the region of 540-570nm).

We have now utilized this absorbance increase for determining stoichiometry by using a modified Job’s plot and optimal conditions for binary (1:1) AA.Cr synthesis. The formation of Phe.Cr is accompanied by a parallel and progressive loss of absorbance of its aromatic peak (\(\varepsilon_{\text{max}}=257\)nm); from this also \(t_{1/2}\) for formation was derived to be 6-7', a value that is identical to that got from \(\Delta A_{570}\), validating the procedure.

The use of chromium sulphate, in place of CrCl\(_3\) used by earlier workers, as the source of Cr\(^{3+}\) to form AA.Cr has been useful. Chromium sulphate has two \(\tilde{\nu}\) (s=\(\delta\)) vibrational peaks at 1137 cm\(^{-1}\) and at 1040 cm\(^{-1}\) These are found in Phe.Cr as well. In Lys.Cr and Gln.Cr, on the other hand, they are replaced by a single peak at 1129 cm\(^{-1}\); clearly sulphate is structurally part of these two complexes but not of Phe.Cr. We may therefore designate them as “sulphato” complexes. However, whether the sulphate bonding is monodentate or bidentate is not clear since IR spectra cannot resolve this issue. (15, 16).

The reactive chromium species obtaining in these studies is hexaaquo chromium. Accordingly and from the physiochemical characteristics of these complexes, the structures of AA.Cr would be those shown in (Fig7). Galuszka et al., (3) have also suggested similar unidentate carboxyl binding for their monomeric complexes.
GTF activity of AA.Cr: The first report of a synthetic amino acid chromium complex with GTF activity is that of (D-Phe)₃.Cr synthesized by Yang et al., (7). However, this is not a binary complex of the AA-Cr (1:1) type which would be the simplest model compound. Our report (13) that 10mM.Gln.Cr shows powerful GTF-like activity in *S. cerevisiae* (NCIM 3558) is the very first report of this kind. We have now found that the minimal effective level is 1mM and Gln.Cr and Phe.Cr but not Lys.Cr considerably enhance the glucose utilization rate, with Gln.Cr. > Phe.Cr. Preliminary experiments have shown recently that Cys.Cr and Gly.Cr also enhance glucose metabolic rates by this Yeast strain and that all four AA.Cr are also active in rats in the critical test of GTF activity, namely the Oral Glucose Tolerance Test (OGTT). (Karthikeyan et al, unpublished) This leads to the hypothesis that the critical determinants of GTF activity are binary AA.Cr complexes.

Metabolism of AA.Cr in Yeast: The first indication that AA.Cr can have hitherto unrecognized roles in metabolism has been the finding that lysine, which cannot be utilized as a nitrogen source by yeast is excellently metabolized, if complexed to trivalent chromium (8).

Present work reveals that such complexation also abolishes the inhibitory effect of lysine on P.E. growth of Yeast. The mechanism involved is unclear. However P.E. growth is known to be due to increase in cell mass due to synthesis of structural polysaccharides but without further cell division(11) and it is clear that Lys.Cr, although ineffective in GTF-like enhancement of rate of the rate of glycolysis, does stimulate the synthetic processes of P.E. growth. As regards Phe.Cr, results of the present study show that complexation of phenylalanine leads to GTF-like activity as well as improved rate of metabolism of its carbon skeleton although not of the amino-N of this amino acid.

Since little is known of the metabolism of binary AA.Cr complexes on yeast, these diverse effects brought to light by the studies herein emphasize that trivalent chromium profoundly modifies the metabolic properties and effects of amino acids to which it binds. In some cases this leads to GTF-active complexes. The finding of highly effective AA.Cr or combinations thereof would be of great importance in developing useful, auxiliary therapeutic agents for diabetes.

Conclusion

A new, general procedure has been devised for the synthesis of binary Amino acid – Chromium complexes and their structures have been defined. The metabolic effects of the complexation of lysine are profound changes in the metabolism of the amino acid and its amino group. With phenylalanine, complex formation positively affects metabolism of its carbon skeleton. Chromium complexes of phenylalanine as well as glutamine exhibit GTF activity and enhance rate of glucose metabolism. The synthetic procedure developed will be useful for making chromium complexes that could be clinically important in regulating glucose metabolism in diabetes.

Acknowledgement

The authors would like to thank the Dept. of Biotechnology, Govt. of India for financial support and for grant of Research Associateship to the first author. Dr. K.S. Karthikeyan would also like to thank his mother Mrs. K. Rajalaxmi Sastry for all her moral support & encouragement.

References


Karthikeyan et al


