Synthetic Multinuclear Chromium Assembly Activates Insulin Receptor Kinase Activity: Functional Model for Low-Molecular-Weight Chromium-Binding Substance

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Received May 14, 1997[⊗]

The biologically-active form of chromium, low-molecular-weight chromium-binding substance (LMWCr), activates the insulin-dependent tyrosine protein kinase activity of insulin receptor (IR). The site of activation was shown to be on the active site fragment of the β subunit of IR. As LMWCr previously has been proposed to contain a multinuclear chromic assembly, the ability of multinuclear chromium assemblies to activate IR kinase activity has been probed. The trinuclear cation $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ (**1**) has been found to activate IR activity in a fashion almost identical to that of LMWCr using rat adipocytic membrane fragments and an active site fragment of IR, while a variety of other chromic complexes have in contrast been found to be ineffective or to inhibit kinase activity. The activation of IR kinase activity by complex **1**, its stability in aqueous and strongly acidic solution**,** and its low molecular weight suggest that it potentially could be used in a treatment for adult-onset diabetes.

Introduction

The biologically-active, naturally-occurring oligopeptide low-molecular-weight chromium-binding substance (LMWCr) has been found to activate the insulin-dependent tyrosine protein kinase activity of insulin receptor (IR) approximately 8-fold with a dissociation constant of circa 250 pM.[1] This activity is directly proportional to the Cr content of the oligopeptide (being maximal at four chromic ions per oligopeptide), while substitution of chromium with metal ions commonly associated with biological systems results in inactiving the oligopeptide. Similarly, LMWCr has been reported to activate a membrane-associated phosphotyrosine phosphatase; this activation also requires four chromic ions per oligopeptide to be maximal, while chromic ions could not functionally be replaced with other transition metal ions.[2] A role for LMWCr in amplification of insulin-signaling has been postulated.[1,3] Chromium is mobilized from the blood and taken up by insulin-dependent cells in response to insulin.[4] LMWCr is maintained in its apo form[5] but possesses a large chromic ion binding constant(s) as it is capable of removing chromium from Cr-transferrin.[5,6] The holoLMWCr is then capable of stimulating IR kinase activity, amplifying the signal of insulin into the insulin-dependent cells. An association between chromium and insulin-dependent glucose and lipid metabolism has been reported for nearly four decades;[7] however, only recently since procedures for isolation of quantities of LMWCr suitable for kinetic and spectroscopic studies have been developed[3] has progress been made in understanding the association on a molecular level.

An association between the essential nutrient chromium and adult-onset diabetes has also been postulated.[8] Most recently Anderson and co-workers found improved glycemic control for 180 adult-onset diabetic patients following chromium supplementation,[9] while Ravina and Slezack using 138 adult-onset diabetic patients found reduced insulin requirements.[10] Unfortunately, the form of chromium used as a dietary supplement in these studies, chromium(III) picolinate, has been found to cause chromosome damage.[11] This suggests that a new form of chromium for use as a dietary supplement and as part of a potential treatment for adult-onset diabetes is required. LMWCr would appear to be a possibility. It has a high LD_{50}[6] and is biologically active, opposed to chromium picolinate and glucose tolerance factor (a material isolated from acid-hydrolyzed Brewer's yeast extracts) which serve only as sources of readily absorbable chromium.[12] [Despite the apparent significance of Cr, as much as ninety percent of the American population and half of the population of developed nations fail to intake the daily recommended safe and adequate quantities of Cr].[13] However, LMWCr is susceptible to hydrolysis under acidic conditions[14] and consequently could not be taken orally without degradation. Herein is reported a synthetic chromium(III) complex which is biologically active, stable under acidic conditions, and readily and inexpensively synthesized and, therefore, may have potential as a new agent in the treatment of adult-onset diabetes and associated conditions and as a nutritional supplement.

Materials and Methods

LMWCr, $[Cr_3O(O_2CCH_3)_6(H_2O)_3]Cl$, and $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]NO_3$. LMWCr was available from previous work and contained

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1997.

(1) Davis, C. M.; Vincent, J. B. *Biochemistry* **1997**, *36*, 4382.
(2) Davis, C. M.; Sumrall, K. H.; Vincent, J. B. *Biochemistry* **1996**, *35*, 12963.
(3) Davis, C. M.; Vincent, J. B. *Arch. Biochem. Biophys.* **1997**, *399*, 335.
(4) Morris, B. W.; Blumsohn, A.; McNeil, S.; Gray, T. A. *Am. J. Clin. Nutr.* **1992**, *55*, 989. Morris, B. W.; Gray, T. A.; MacNeil, S. *Clin. Sci.* **1993**, *84*, 477. Morris, B. W.; MacNeil, S.; Stanley, K.; Gray, T. A.; Fraser, R. *J. Endrocrin.* **1993**, *139*, 339.
(5) Yamamoto, A.; Wada, O.; Ono, T. *Eur. J. Biochem.* **1987**, *165*, 627.
(6) Yamamoto, A.; Wada, O.; Ono, T. *J. Inorg. Biochem.* **1984**, *22*, 91.
(7) Mertz, W.; Roginski, E. E.; Schwartz, K. *J. Biol. Chem.* **1961**, *236*, 318. Mertz, W.; Roginski, E. E. *J. Biol. Chem.* **1963**, *238*, 868. Mertz, W. *J. Nutr.* **1993**, *123*, 626. Vincent, J. B. In *Encyclopedia of Inorganic Chemistry*; King, B., Ed.; John Wiley: New York, 1994; Vol. 2, pp 661−665.
(8) Anderson, R. A. *Biol. Trace Elem. Res.* **1992**, *32*, 19.
(9) Anderson, R. A.; Cheng, N.; Bryden, N.; Polansky, M.; Cheng, N.; Chi, J.; Feng, J. *Diabetes* **1996**, *45*, Suppl. 2, 124A.
(10) Ravina, A.; Slezack, L. *Harefuah* **1993**, *125*, 142.
(11) Stearns, D. M.; Wise, J. P., Jr.; Patierno, S. R.; Wetterhahn, K. E. *FASEB J.* **1995**, *9*, 1643.
(12) McCarty, M. F. *J. Opt. Nutr.* **1993**, *2*, 36. Vincent, J. B. *J. Nutr.* **1994**, *124*, 117.
(13) Anderson, R. A.; Kozlovsky, A. S. *Am. J. Clin. Nutr.* **1985**, *41*, 1177. Anderson, R. A. In *Risk Assessment of Essential Elements*; Mertz, W., Abernathy, C. O., Olin, S. S., Eds.; ISLI Press: Washington, DC, 1994; pp 187−196.
(14) Sumrall, K. H.; Vincent, J. B. *Polyhedron,* in press.

S0020-1669(97)00568-5 CCC: $14.00

3.6−4.0 chromium per oligopeptide.[1−3] $[Cr_3O(O_2CCH_3)_6(H_2O)_3]Cl$ (**2**) and $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]NO_3$ (**1**) were prepared as previously described.[15,16] Oligopeptide concentrations were assayed using the fluorescamine procedure of Undenfriend and co-workers[17] with glycine as a standard. Chromium concentrations were measured using the diphenylcarbazide method[18] and the method of standard additions to minimize any potential matrix effects. For all kinetic experiments, solutions of LMWCr, **1**, and **2** were prepared by dilutions from more concentrated stock solutions. The chromium(III)−amino acid mixture was prepared by mixing chromium(III) nitrate nonahydrate, aspartate, glutamate, glycine, and cysteine in a 4:2:4:2:2 ratio in water, followed by heating at 37 °C for 30 min to allow complexes to form. To obtain proper concentrations of this mixture (in terms of Cr concentration) for kinetic experiments, serial dilutions were prepared.

Purification of Isolated Adipocytes and Adipocytic Membranes. Fat cells from male Sprague Dawley rats were isolated using the procedures of Rodbell[19] and Anderson et al.[20] with modifications.[2] Three rats (not kept on a Cr-deficient diet) were sacrificed by decapitation and their epididymal fat pads removed. Rat adipocytes were washed with 1% bovine serum albumin (BSA), 50 mM Hepes, pH 7.4 buffer containing 10 μg/mL leupeptin and 5 μg/mL aprotinin. Cells were homogenized with a manual Teflon homogenizer and frozen and thawed five times. The lipid layer was removed, and the cell homogenate was centrifuged for 1 h at 40 000g. The supernatant was removed, and the pellet was suspended in Hepes buffer and used as the source of membrane phosphatase and kinase activity. Protein concentrations were analyzed using the BCA method (Pierce Chemical Co.) with BSA as standard.

Phosphotyrosine Phosphatase Activity. *p*-Nitrophenyl phosphate (*p*-NPP) was used to determine the amount of PTP activity using the method of Li et al.[21] The assay used 5 mM substrate in 0.05 M Tris, pH 7.5, unless noted. Activation of PTP activity by LMWCr and other Cr-containing species was examined as described by Davis et al.[2] Solutions of LMWCr, **1**, **2**, and the Cr−amino acid mixture were incubated with the enzyme for 15 min at 37 °C before initiation of the reaction. Hydrolyses proceeded for 1 h at 37 °C. The extent of hydrolysis was determined at 404 nm ($\epsilon = 1.78 \times 10^4$ M^{-1} cm^{-1}).

Phosphotyrosine Kinase Activity. Phosphotyrosine kinase activity was measured using a protein tyrosine kinase assay kit (Boehringer Mannheim) which uses an anti-phosphotyrosine antibody to recognize phosphotyrosine. A fragment of gastrin (amino acids 1−17) which has been biotinylated so it can be immobilized to streptavidin-coated microtiter plates (Boehringer Mannheim) was used as the substrate. The assays were performed in 50 mM Tris, pH 7.4 containing 0.75 μM ATP and 7.5 mM $MgCl_2$ at 37 °C for 75 min as previously described[1] unless otherwise noted. The membrane fragments which contain phosphotyrosine themselves were removed after the reaction was terminated with EDTA by Microcon 30 or Microcon 50 microconcentrators (Amicon), and ammonium vanadate was used as a phosphatase inhibitor. Contributions to the assay by the addition of metal-containing materials were determined by measuring the background absorbance of the assay in the absence of membranes, and these contributions were subtracted from all data. Bovine pancreas insulin was from Sigma. Isolated kinase active site fragment from the β subunit of human insulin receptor (residues 941−1343) was obtained from Stratagene and diluted with 50 mM Tris, pH 7.4; the fragment does not require activation of the kinase activity by added insulin. Five units of IR fragment were used in kinetic assays; a unit of activity is defined as the picomoles of (phosphate incorporated/min)/μL of kinase as received from the manufacturer. Recombinant human insulin-like growth factor-1 (IGF-1) was obtained from Sigma and reconstituted with 10 mM HCl.

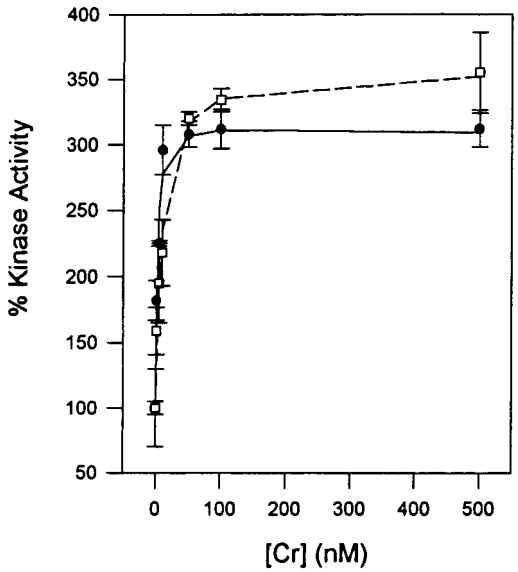

Figure 1. Activation of protein tyrosine kinase activity of the isolated active site fragment of the β subunit of insulin receptor by bovine liver LMWCr (open squares) and $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]NO_3$ (solid circles) using a fragment of gastrin (0.75 mM) as substrate. The line is the best curve fit giving for LMWCr a dissociation constant of 133 pM and for $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ a dissociation constant of 1.00 nM.

Miscellaneous. All visible spectroscopic measurements were obtained with a Shimadzu UV-160A diode array spectrophotometer. Fluorescence measurements were made with a Perkin-Elmer 204 fluorescence spectrophotometer. ^1H NMR were obtained using a Bruker AM-360 spectrometer at circa 23 °C. Chemical shifts are reported on the δ scale (shifts downfield are positive) using solvent protio impurity as a reference. Curve-fitting was performed using SigmaPlot (Jandel Scientific). All kinetic experiments were performed in triplicate. Errors are presented throughout including all tables and graphs as the standard deviations (1σ) of the triplicate analyses. Similarly, all Cr and LMWCr concentration determinations were made in triplicate. Doubly deionzed water was used in all operations; plasticware was used whenever possible.

Results and Discussion

The binding of insulin to the α subunit of insulin receptor results in tyrosine autophosphorylation of the β subunit of the receptor, transmitting the signal of the hormone insulin into a cell; autophosphorylation activates the kinase in the β subunit which catalyzes phosphorylation of other proteins.[22] This kinase activity is potentiated by the oligopeptide LMWCr. Using isolated IR, potentiation of IR tyrosine protein kinase activity by LMWCr has been found to require insulin and is prevented when the insulin binding site of the external α subunit is blocked.[1] However, the binding site on IR for LMWCr is unknown. However, studies with a catalytically active fragment (residues 941−1343) of the β subunit of the human enzyme indicate that the effect of LMWCr on kinase activity is associated with this fragment. As shown in Figure 1, addition of LMWCr to the fragment results in an approximately 3-fold activation of the kinase activity. Fitting the curve to a hyperbolic equation gives a dissociation constant for LMWCr of 133 pM, very similar to the dissociation constant found for the interaction of LMWCr with isolated rat insulin receptor (250 pM).[1] The 3-fold activation is significantly less than that observed with isolated receptor (approximately 8-fold),[1] but this may be associated with small structural differences between the

(15) Johnson, M. K.; Powell, D. B.; Cannon, R. D. *Spectrochim. Acta* **1981**, *37A*, 995.
(16) Earnshaw, A.; Figgis, B. N.; Lewis, J. *J. Chem. Soc. A* **1966**, 1656.
(17) Undenfriend, S.; Stein, S.; Bohlen, P.; Dairman, W.; Leimgruber, W.; Weigele, M. *Science* **1972**, *178*, 871.
(18) Marczenko, Z. *Spectrophometric Determination of the Elements*; Ellis Horwood: Chichester, England, 1986.
(19) Rodbell, M. *J. Biol. Chem.* **1964**, *239*, 375.
(20) Anderson, R. A.: Brantner, J. H.; Polansky, M. M. *J. Agric. Food Chem.* **1978**, *26*, 1219.
(21) Li, J.; Elberg, G.; Gefel, D.; Shechter, Y. *Biochemistry* **1995**, *34*, 6218.
(22) Lee, J.; Pilch, P. F. *Am. J. Physiol.* **1994**, *35*, C319. White, M. F.; Kahn, C. R. *J. Biol. Chem.* **1994**, *269*, 1.

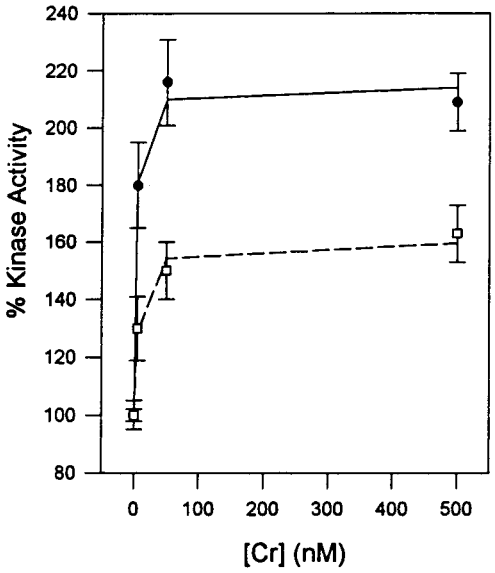

Figure 2. Activation of rat adipocytic membrane protein tyrosine kinase activity using a fragment of gastrin (0.75 mM) as substrate by LMWCr (solid circles) and by $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]NO_3$ (open squares) in the presence of 100 nM IGF-1. A 25 μL volume of a rat membrane suspension corresponding to 0.0895 mg of protein/mL was utilized. The line is the best fit hyperbolic curve giving a dissociation constant of 507 pM for LMWCr and for $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]$-$NO_3$ a dissociation constant of 730 pM.

fragment and the entire receptor protein. The results suggest that LMWCr may associate with the kinase active site of the insulin receptor.

Insulin receptor is part of a family of receptor proteins which includes the insulin-like growth factor receptors.[23] All these receptors are disulfide-bound heterotetramers of α and β subunits. Ligand (insulin or insulin-like growth factors) presumably cause a conformational change in the preformed receptors, resulting in receptor activation. To examine the specificity of LMWCr, the effects of the oligopeptide on IGF-1 receptors were probed. The kinase activity of rat adipocytic membrane fragments in the presence of 100 nM IGF-1 is more than doubled by the addition of LMWCr (Figure 2) with a dissociation constant of 507 pM. In contrast, in the presence of 100 nM insulin kinase activity has previously been shown using the same fragment of gastrin as substrate to be increased three and one-half times by LMWCr with a similar dissociation constant of 875 pM.[1] [In the absence of added hormone, LMWCr has no detectable effect on the membrane kinase activity.[1]] Thus, LMWCr potentiates both members of the IGF receptor family.

Given this novel role in the amplification of signal transduction for LMWCr and its rather simple composition (carboxylate-rich oligopeptide binding four chromic ions),[3,5] the possibility of identifying a functional model for LMWCr was examined. Such a model would be required to be soluble and stable in aqueous solution, be well characterized, and contain a carboxylate-supported multinuclear chromic assembly.[3] Fortunately, a review of the literature revealed a number of trinuclear and tetranuclear Cr(III) carboxylate assemblies;[24,25] however, few

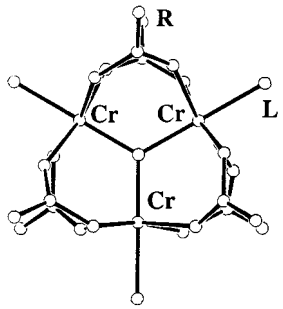

Figure 3. Structure of oxo-centered trinuclear chromium carboxylate cations: Compound **1**, L = H_2O, R = CH_2CH_3; compound **2**, L = H_2O, R = CH_3.

were soluble in water. On the basis of these requirements, two were chosen: $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ (**1**) and $[Cr_3O(O_2$-$CCH_3)_6(H_2O)_3]^+$ (**2**). Both of these complexes possess a basic carboxylate type structure[25] comprised of a planar triangle of chromic ions with a central μ_3-oxide (Figure 3). Each set of two chromic ions is bridged by two carboxylates ligands, while six coordination about the chromium centers is completed by a terminal aquo ligand.

The cation $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ (**1**) is a well-characterized species. Its preparation was first described in 1911, although the formula was proposed as a hydrate salt of $[Cr_3(O_2CCH_2CH_3)_6(OH)_2]^+$.[26] A similar synthesis of a variety of salts of the cation (still with the wrong formulation) was reported in 1930.[27] The cation, which was originally characterized only by its color and elemental analysis, has subsequently been characterized by variable-temperature magnetic susceptibility measurements,[16,28] electronic spectroscopy,[28] luminescence spectroscopy,[29] infrared spectroscopy and X-ray crystallography (of the nitrate salt),[30] ESR,[31] fast atom bombardment and electrospray mass spectrometry,[32] and NMR.[33] The cation $[Cr_3O(O_2CCH_3)_6(H_2O)_3]^+$ (**2**) has been more exhaustively studied and has served as a model upon which theories of the magnetic interactions between multiple paramagnetic centers were tested (reviewed in ref 25).

The ability of the synthetic materials to activate membrane phosphotyrosine protein phosphatase activity and insulin-dependent membrane tyrosine protein kinase activity was examined (LMWCr has previously been shown to also activate a membrane-associated phosphotyrosine phosphatase activity in rat adipocytic membrane fragments).[2] As shown in Figures 4 and 5, the acetate triangle **2** does not activate but rather inhibits both the membrane phosphatase and kinase activity. In stark contrast, the propionate analogue results in an *activation* of both activities in a fashion very similar to LMWCr. The kinase activity is stimulated approximately 2-fold, while the phosphatase activity is increased nearly 50%. Fitting the curves of Figures 4 and 5 to a hyperbolic function results in dissociation constants for the trinuclear species of 2.98 and 30 nM for the

(23) Heldin, C.-H. *Cell* **1995**, *80*, 213.
(24) (a) Harton, A.; Nagi, M. K.; Glass, M. M.; Junk, J. L.; Atwood, J. L.; Vincent, J. B. *Inorg. Chim. Acta* **1994**, *217*, 171. (b) Ellis, T.; Glass, M.; Harton, K.; Huffman, J. C.; Vincent, J. B. *Inorg. Chem.* **1994**, *33*, 5522. (c) Nagi, M. K.; Harton, A.; Donald, S.; Lee, Y. S.; Sabat, M.; O'Connor, C. J.; Vincent, J. B. *Inorg. Chem.* **1995**, *34*, 3813. (d) Bino, A.; Chayat, R.; Pederson, E.; Schneider, A. *Inorg. Chem.* **1991**, *30*, 856. (e) Donald, S.; Terrell, K.; Vincent, J. B.; Robinson, K. D. *Polyhedron* **1995**, *14*, 971.
(25) Cannon, R. D.; White, R. P. *Prog. Inorg. Chem.* **1988**, *36*, 195.
(26) Weinland, R. F.; Hoehn, K. *Z. Anorg. Chem.* **1911**, *69*, 158.
(27) Weinland, R. F.; Lindner, J. *Z. Anorg. Chem.* **1930**, *190*, 285.
(28) Szynanska-Buzar, T.; Ziolkowski, J. J. *Sov. J. Coord. Chem.* **1976**, *2*, 897. Zelentsov, V. V.; Zhemchuzhikova, T. A.; Rakitin, Yu. V.; Yablokov, Yu. V.; Yakubov, Kh. M. *Koord. Khim.* **1975**, *1*, 194. Yoshida, T.; Morita, M.; Date, M. *J. Phys. Soc. Jpn.* **1988**, *57*, 1428.
(29) Morita, M.; Kato, Y. *Int. J. Quantum Chem.* **1980**, *18*, 625.
(30) Antsyshkina, A. S.; Porai-Koshits, M. A.; Arkhangel'skii, I. V.; Diallo, I. N. *Russ. J. Inorg. Chem.* **1987**, *32*, 1700.
(31) Hondo, M.; Morita, M.; Date, M. *J. Phys. Soc. Jpn.* **1992**, *61*, 3773. Nishimura, H.; Date, M. *J. Phys. Jpn.* **1985**, *54*, 395.
(32) Fu, G.; Yu, L.; Zhu, Z.; Xie, W.; Zheng, Y.; Zhang, L. *Jiegou Huaxue* **1990**, *4*, 278. van den Bergen, A.; Colton, R.; Percy, M.; West, B. O. *Inorg. Chem.* **1993**, *32*, 3408.
(33) Glass, M. M.; Belmore, K.; Vincent, J. B. *Polyhedron* **1993**, *12*, 133.

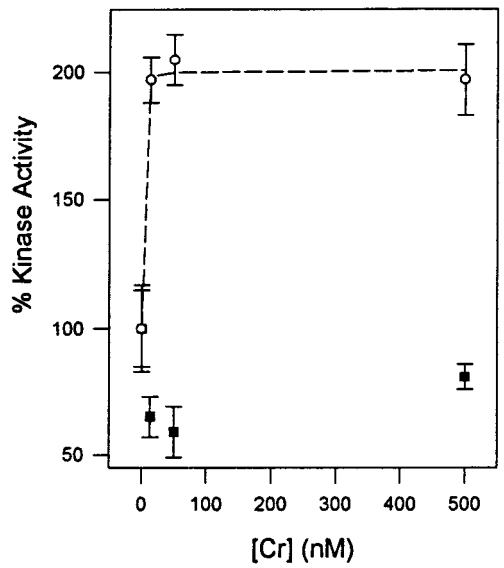

Figure 4. Activation of rat adipocytic membrane protein tyrosine kinase activity using 0.75 mM gastrin (amino acids 1−17) as substrate by $[Cr_3O(O_2CCH_3)_6(H_2O)_3]Cl$ (solid squares) and by $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]NO_3$ (open circles) in the presence of 100 nM insulin. A 5 μL volume of rat adipocyte membrane suspension corresponding to 0.0856 mg of protein/mL was utilized. The 100% activity corresponds to insulin-stimulated kinase activity and is typically about 0.338 pmol of phosphotyrosine/mg of membranes. The line is the best fit hyperbolic curve yielding a dissociation constant of 2.98 nM for $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]NO_3$.

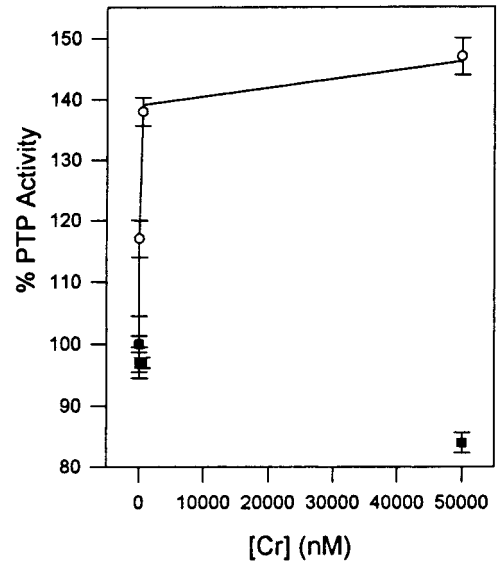

Figure 5. Activation of rat adipocytic membrane protein phosphatase activity using 5 mM *p*-NPP as substrate by $[Cr_3O(O_2CCH_3)_6(H_2O)_3]Cl$ (solid squares) and by $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]NO_3$ (open circles). A 125 μL volume of a rat membrane suspension corresponding to 0.0856 mg of protein/mL was utilized. The line is the best fit hyperbolic curve yielding a dissociation constant of 30 nM for $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$.

kinase and phosphatase activities, respectively. These results are strikingly similar to those using LMWCr. LMWCr results in a 250% increase in insulin-dependent tyrosine kinase activity with a dissociation constant of 875 pM[1] (one third that of the model) and a 100% increase in phosphatase activity with a dissociation constant of 4.4 nM[2] (one-seventh that of the model). Consequently, **1** is an excellent functional model of LMWCr but possesses somewhat less activation while requiring slightly higher concentrations to achieve these affects. To test just how good a model of LMWCr that complex **1** is, its ability to activate the active site fragment of the β subunit of IR and the IGF-1 receptor were also examined (Figures 1 and 2). For the IR β subunit fragment, complex **1** resulted in a circa 60% increase in kinase activity with a dissociation constant of 730 nM; for the IGF-1-dependent membrane kinase activity, an increase of 250% was observed with a dissociation constant of 1.00 nM. In both cases the dissociation constant for the synthetic material is within 1 order of magnitude of that for LMWCr. Thus, the trinuclear chromic assembly **1** mimics LMWCr in its ability to activate adipocytic membrane phosphotyrosine phosphatase activity, insulin-dependent adipocytic membrane tyrosine protein kinase activity, insulin-like growth factor-1-dependent adipocytic membrane tyrosine protein kinase activity, and the tyrosine protein kinase activity of the active site fragment of the β subunit of insulin receptor. The ability of LMWCr and complex **1** to activate both protein tyrosine kinases and phosphotyrosine phosphatases may seem paradoxical; however, the stimulation of both types of enzymes appears to be common in complex signal transduction pathways.[34]

To guarantee that the trinuclear cation **1** was the actual active species in solution, the stability of complex in water and in the buffer had to be ascertained. Paramagnetic NMR has been demonstrated to be of utility in characterizing antiferromagnetically-coupled chromium(III) assemblies.[24a−c,33,35] For acetate and propionate ligands bridging between chromic centers in these assemblies, the resonances of methyl hydrogens of acetate and the methylene protons of propionate occur in the +35 to +45 ppm range.[33] The nitrate salt of **1** was dissolved in D_2O and in 50 mM Tris buffer (prepared by dissolving Tris in D_2O and adding a quantity of DCl equivalent to the quantity of HCl needed to make the same quantity of 50 mM Tris buffer, pH 7.4 in H_2O); ^1H NMR spectra of 5 mM solutions of **1** were collected every 5 min for 2 h. During this period, the integrations of the propionate methylene resonances (+42 ppm) were unchanged, and no new signals appeared. Thus, the triangle appears to be stable in aqueous solution and in the assay buffer, and the activation activity can be assigned to the trinuclear cation.

Additionally the components of **1** were examined for any ability to potentiate membrane phosphatase activity and insulin-dependent membrane kinase activity (Figure 6). Propionic acid and nitrate (at concentrations comparable to those if **1** dissociated completely in water) do not potentiate either phosphatase or kinase activity; previously, mononuclear chromic salts were also shown to not result in potentiation of either activity.[1,2] A mixture of the components of LMWCr was similarly tested for its ability to potentiate these activities. The mixture consisted of chromic ions, aspartate, glutamate, glycine, and cysteine in a 4:2:4:2:2 ratio, corresponding to the approximate ratio of the components in isolated bovine liver LMWCr.[3] At a chromium concentration equivalent to that used for LMWCr in the assays, the mixture actually inhibited kinase and phosphatase activity. Thus, the multinuclear chromic complexes **1** and LMWCr appear to be unique in their ability to potentiate membrane phosphotyrosine phosphatase and insulin-dependent membrane kinase activity. Unfortunately, the isostructural Mn(III) and Fe(III) analogues of complexes **1** and **2** are unstable in water and consequently unsuitable for use for comparison in the phosphatase and kinetic assays.

While LMWCr has been proposed for use as a nutritional supplement and in treatment of adult-onset diabetes and related conditions associated with improper carbohydrate and lipid

(34) Hunter, T. *Cell* **1995**, *80*, 225.
(35) Vincent, J. B. *Inorg. Chem.* **1994**, *33*, 5604. Belmore, K.; Madison, X. J.; Harton, A.; Vincent, J. B. *Spectrochim. Acta* **1994**, *50A*, 2365.

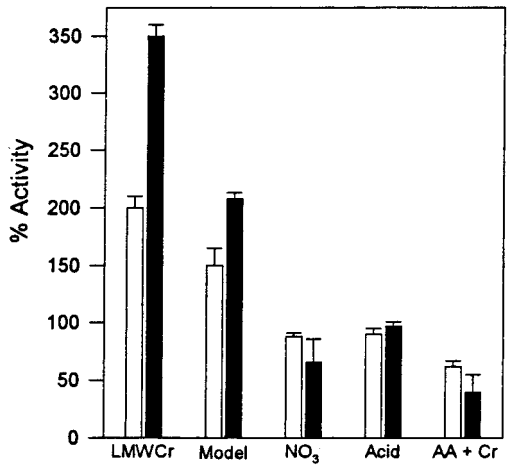

Figure 6. Influence of chromium complexes and their components on adipocytic membrane tyrosine protein kinase activity in the presence of 100 nM insulin (solid bars) and adipocytic membrane phosphotyrosine phosphatase activity (open bars). The 100% activity represents the activity in the absence of added chromium complexes or their components. A 25 μL volume of a rat membrane suspension corresponding to 0.0895 mg of protein/mL was utilized. For the kinase assays, 50 nM concentrations of LMWCr, complex **1**, and the Cr−amino acid mixture (in terms of Cr) were used; 500 nM nitrate and propionate were used. For the phosphatase assays, 50 μM concentrations of LMWCr, complex **1**, and the Cr−amino acid mixture (in terms of Cr) were used; 50 μM nitrate and propionate were used. Model = complex **1**; Acid = propionic acid; AA + Cr = chromium−amino acid mixture.

metabolism,[1−3] complex **1** may be even more promising for use in these applications. The synthetic material is prepared from inexpensive reagents[16] (and consequently not requiring a time-consuming isolation as with LMWCr), is extremely stable in aqueous solution (LMWCr undergoes a slow hydrolysis),[3] and even stable in acidic solution. The trinuclear basic carboxylates of chromium(III) can, for example, be recrystallized from dilute mineral acids;[15] consequently **1** might readily survive oral ingestion unlike LMWCr. Cation **1** also has a molecular weight of 664 compared to approximately 1480 Da for bovine liver LMWCr,[3] which should facilitate movement of the former across cell membranes. Curiously, Mirsky and co-workers reported that addition of cations **1** and **2** and $[Cr_3O(O_2CH)_6(H_2O)_3]^+$ caused a 15−20% enhancement of carbon dioxide production by yeast;[36] the similar behavior of the three cations does not agree with the quite different behavior seen for cations **1** and **2** in this work. Unfortunately, experimental details for the studies with the three cations by Mirsky et al. are lacking, and no data are presented. As a result, it is difficult to determine how the cations might be influencing fermentation and to ascertain the relationship (if any) between the yeast fermentation assay and human glucose and lipid metabolism.

Conclusions

These studies show that LMWCr and the synthetic analogue $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ activate insulin receptor protein tyrosine kinase activity by interacting at or near the kinase active site of the enzyme's β subunit, while both materials are able to activate protein tyrosine kinase activity of rat adipocytic membrane fragments in response to insulin-like growth factor in addition to insulin and also activate phosphotyrosine phosphatase activity of adipocytic membranes. The similarity between the activation by LMWCr and complex **1** supports the proposal that LMWCr possesses a multinuclear chromic assembly similar to that of complex **1**.[3] The mechanism of the activation by the chromium complexes is under investigation, as are the effects of the materials on diabetic animal models.

Acknowledgment. The authors gratefully acknowledge the support of this work by the American Heart Association (Grant-in-Aid 94011190).

IC970568H

(36) Mirsky, N.; Weiss, A.; Dori, Z. *J. Inorg. Biochem.* **1980**, *13*, 11.