0026-895X/00/040738-09$3.00/0
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 58:738–746, 2000

Vol. 58, No. 4
172/855373
Printed in U.S.A.

Organic Vanadium Chelators Potentiate Vanadium-Evoked Glucose Metabolism In Vitro and In Vivo: Establishing Criteria for Optimal Chelators

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Received March 23, 2000; accepted July 3, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

Several ligands, when complexed with vanadium, potentiate its insulinomimetic activity both in vivo and in vitro. We have recently found that L-Glu-γ-monohydroxamate (HXM) and L-Asp(β)HXM were especially potent in this regard. In the present study, we used vanadium-enriched adipose cells and cell-free experimental systems to determine the features of L-Glu(γ)HXM and L-Asp(β)HXM that turn these ligands into optimal-synergizing vanadium chelators. We found that L-Glu(γ)HXM and L-Asp(β)(HXM) possess the following characteristics: 1) They associate with vanadium(+5) at pH 7.2 within a narrow range of an apparent formation constant of 1.3 to 1.9×10^2 M^{-1}; 2) they have nearly the same binding affinity for the vanadyl(+4) cation and the vanadate(+5) anion at physiological pH values; and 3) they form intense ultraviolet absorbing complexes upon associating with vanadium(+4) at 1 and 3 M stoichiometry, respectively, at pH 3.0. Vanadium ligands lacking any of these three defined criteria synergize less effectively with vanadium to activate glucose metabolism.

Vanadium salts mimic the actions of insulin in vitro (Shechter and Karlish, 1980; Shechter, 1990; Shechter et al., 1995). In diabetic rats, vanadium therapy induces normoglycemia and improves glucose homeostasis in insulin-deficient and -resistant diabetic rodents (Meyerovitch et al., 1987; Brichard and Henquin, 1995). Accumulating data suggest that vanadium acts through *alternative* noninsulin pathways, involving inhibition of protein phosphotyrosine phosphatases and activation of nonreceptor protein tyrosine kinases (Green, 1986; Fantus et al., 1989; Mooney et al., 1989; Strout et al., 1989; Shisheva et al., 1991; Venkatesan et al., 1991; Shisheva and Shechter, 1992, 1993; Elberg et al., 1994, 1997). Accordingly, vanadium salts are effective in diabetic rodents that do not respond at all to insulin (reviewed in Brichard and Henquin, 1995).

Initial clinical studies have already been performed with low doses of vanadium (2 mg/kg/day) to avoid toxicity. Although 10- to 20-fold lower than those used in most animal studies, even these doses of vanadium yielded several beneficial effects (Cohen et al., 1995; Goldfine et al., 1995; Halberstam et al., 1996). Any manipulation that elevates the insulinomimetic efficacy of vanadium without increasing its toxicity is of significant potential interest for the future care of diabetes in humans.

Various organically chelated vanadium compounds are more potent than free vanadium salts in facilitating insulin-like effects in vitro and in vivo (McNeill et al., 1992; Shechter et al., 1992; Sakurai et al., 1995; Li et al., 1996). Recently, we found that the L-isomer of Glu(γ)monohydroxamate (HXM) is particularly active; in streptozocin (STZ)-rats, L-Glu(γ)HXM-vanadium complex (2:1 stoichiometry) potentiated vanadium-induced normalization of circulating glucose levels by 4- to 5-fold. In rat adipocytes, in vitro, L-Glu(γ)HXM itself, in the absence of vanadium, activated hexose transport and lipogenesis to 20 to 35% of maximal stimulation (Goldwaser et al., 1999). We thus consider L-Glu(γ)HXM an optimal-synergizing vanadium chelator.

In this study, we wished to determine whether the vanadium-enhancing effect of L-Glu(γ)HXM can be attributed to a specific feature or features of this ligand and examine other less active vanadium ligands for these characteristics. To this end, we developed three experimental systems. First, we determined the approximate formation constants of various ligands toward vanadium(+4) and vanadium(+5) at neutral pH. Second, vanadium-enriched adipocytes were exposed to different vanadium ligands to assess their potential lipogenic

This study was supported by grants from the Minerva Foundation, Munich, Germany; The Israel Academy of Science Foundation; the Israeli Ministry of Health; and The Lapid Pharmaceutical Company. Y.S. is the incumbent of C.H. Hollenberg Chair in Metabolic and Diabetes Research, established by the friends and associates of Dr. C.H. Hollenberg of Toronto, Canada. M.F. is the Lester Pearson Professor of Protein Chemistry. This work is in partial fulfillment of the requirements for the Ph.D. degree of I.G.

ABBREVIATIONS: HXM, monohydroxamate; STZ, streptozocin; KRBH, Krebs-Ringer bicarbonate HEPES; pNPP, *p*-nitrophenylphosphate.

activity. Finally, we examined whether a favorable geometry (such as that of vanadium-L-Glu(γ)HXM) may have physicochemical attributes that are reflected by simple spectrophotometric measurements.

Experimental Procedures

Materials. D-[U-^{14}C]Glucose was from New England Nuclear (Boston, MA). Collagenase type I (134 U/mg) was from Worthington Biochemicals (Freehold, NJ). Porcine insulin was from Eli Lilly Co. (Indianapolis, IN).

L-Glu(γ)HXM, L-Asp(β)HXM, and L-Ile(α)HXM were from Sigma Chemical Co. (St. Louis, MO). Vanadyl acetylacetonate was from Aldrich Chemical Co. (Milwakee, WI). Bis-(maltolato)oxovanadium was prepared as described by Caravan et al. (1995), and bis-(picolinato)oxovanadium was prepared as described by Sakurai et al. (1995).

Krebs-Ringer bicarbonate HEPES (KRBH) buffer, pH 7.4, contained 117 mM NaCl, 10 mM $NaHCO_3$, 1 mM CaCl, 1 mM $MgSO_4$, 4 mM KH_2PO_4, and 30 mM HEPES. All other chemicals and reagents used were of analytical grade.

Cell Preparation and Bioassay for Lipogenesis. Rat adipocytes were prepared from fat pads of male Wistar rats (130 to 150 g) by collagenase digestion according to the method of Rodbell (1964). Viability of cell preparations, evaluated by Trypan blue exclusion, was more than 95% at least 3 h after digestion. Assay of lipogenesis (the incorporation of U-^{14}C-labeled glucose into lipids) was performed essentially according to Moody et al. (1974). Briefly, freshly prepared rat adipocytes were suspended in KRBH/0.7% BSA buffer and divided into 50 plastic vials, each containing 0.5 ml of adipocyte suspension (about 1.5×10^5 cells). These were incubated with 0.16 mM [U-^{14}C]glucose at 37°C for 2 h under an atmosphere of 95% O_2/5% CO_2. Each assay was compoed of vials with and without 17 nM insulin and the various test compounds. Lipogenesis was terminated by adding toluene-based scintillation fluid, after which radioactivity was counted in the extracted lipids (Moody et al., 1974). Results are expressed as percentage of maximal insulin response. Only assays in which insulin activated lipogenesis 5- to 6-fold above basal level (~4000 cpm per 1.5×10^5 cells/2 h; V_{ins} = 20,000–24,000 cpm per 1.5×10^5 cells/2 h) were taken into consideration. Insulin activated lipogenesis in this assay at an ED_{50} of 33 ± 0.03 pM. A concentration of 0.3 nM insulin already facilitated maximal (100%) response (Shisheva and Shechter, 1992). All assays were performed in duplicate or triplicate.

Saccharomyces cerevisiae. *S. cerevisiae* (wild type) was obtained from the Weizmann Institute biological services and grown overnight in yeast extract/peptone/dextrose media at 25°C. Before the experiment, cells were suspended and diluted in the same medium, brought to pH 7.4 with NaOH, divided into several vials, and supplemented with none or the indicated concentrations of $NaVO_3$(+5) or $VOCl_2$(+4) in quadruplicate for each measurement. The pH was readjusted to 7.4 (when needed). Cells were then incubated for 5 h at 37°C with moderate shaking. Growth was evaluated by measuring turbidity at 540 nm.

Preparation of Vanadium-Enriched Adipocytes. Male-Wistar rats (7 weeks old, 140 ± 10 g) were s.c. injected daily with $NaVO_3$ (0.1 mmol/kg at 10:00 AM) over a period of 4 days. The rats were sacrificed by decapitation 4 to 6 h after the last injection. Epididymal fat pads were removed to determine vanadium levels by atomic emission spectroscopy and prepare rat adipocytes for use in the lipogenic assay. Vanadium-enriched adipocytes prepared in this way had higher basal activity, attributed to the higher level of intracellular vanadium [~8400 ± 300 cpm per 1.5×10^5 cells/2 h, whereas V_{ins} remained nonmodified (V_{ins} = 20,000–24,000 cpm per 1.5×10^5 cells/2 h)]. After enrichment in vivo, the level of vanadium in the adipose tissue was 0.39 ± 0.03 μg/g (~7.65 μM), as determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES model spectroscope, Kleeve, Germany). Using this procedure (sensitivity, 2.55 μM), the vanadium level was undetectable in the adipose tissue of control rats [although others have reported a control level of 0.3 μM (Sitpirija and Eiam-Ong, 1998)]. Thus in vivo enrichment elevated vanadium level about 25-fold.

Evaluating Relative Affinities of Ligands for Vanadium: Reversal of Vanadium-Evoked Inhibition of Acid Phosphatase by Vanadium Chelators. This assay evaluates the efficacy of vanadium chelators by measuring their ability to reverse vanadium-evoked inhibition of acid phosphatase at pH 7.2. The assay is performed in 4-ml plastic tubes (0.5 ml per tube) in 0.05 M HEPES buffer, pH 7.2, containing 1.0 M KCl, 0.2 mM *p*-nitrophenylphosphate (pNPP), either $NaVO_3$ or $VOCl_2$ (final concentration, 20 μM), increasing concentrations of the chelator to be tested, and wheat-germ acid phosphatase (50 μg per tube). pNPP hydrolysis was terminated after 10 min at 25°C by adding NaOH (50 μl of 5 M NaOH per tube), and the absorbency at 410 nm, corresponding to *p*-nitrophenolate, was determined. Because several chelators enhance pNPP-hydrolysis by acid phosphatase, rate of hydrolysis was determined individually for each concentration of chelator in the absence (Vo) and the presence (Vi) of 20 μM $NaVO_3$ or $VOCl_2$. The plot of inhibited-extent of hydrolysis versus the chelator concentration is linear with a slope proportional to the affinity constant of the ligand for vanadium. RC_{50} is defined here as the concentration of the chelator that half-maximally reverses vanadium-evoked inhibition of acid phosphatase. All values are presented as means ± S.D.

Results

Evaluation of the Intact Adipose Cell System as a Possible Measure for Classifying Insulinomimetic-Synergizing Vanadium Ligands. Initially, we examined whether the intact adipose cell was an appropriate experimental system for classifying vanadium ligands in respect to their potentiating effect. Figure 1A shows that 10 μM concentrations of either $VOCl_2$(+4), bis-(picolinato)oxovana-

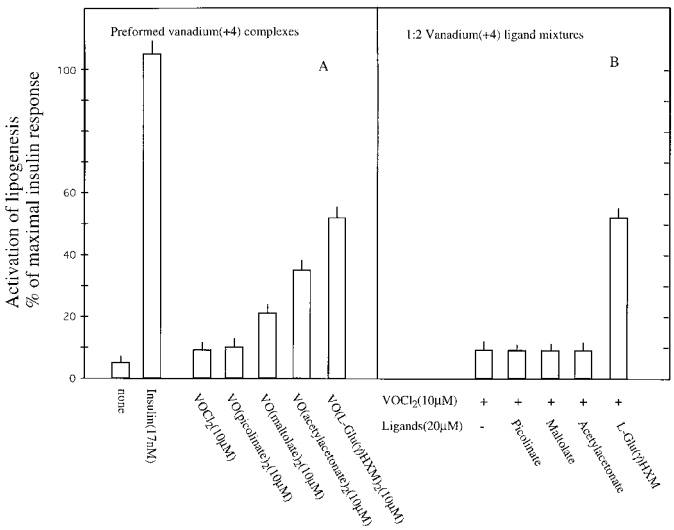

Fig. 1. Lipogenic capacity of low concentration of vanadium(+4), with several organic chelators: comparison between preformed vanadium(+4) complexes and vanadium(+4)-ligand mixtures. A, freshly prepared rat adipocytes (3×10^5 cells/ml) were suspended in KRBH-buffer, pH 7.4, containing 0.7% BSA and preincubated for 10 min with 10 μM $VOCl_2$ and 10 μM concentrations of the preformed organically chelated vanadium(+4) compounds. B, the indicated organic chelators (20 μM) were added first to the adipocytic cell suspension followed by addition of $VOCl_2$ (10 μM) 10 s later. Cells were then supplemented with [U-^{14}C]glucose and lipogenesis was performed for 2 h at 37°C. Radioactivity incorporated into extracted lipids was then determined. The reference maximal response (100%) was that obtained in the presence of 17 nM insulin

dium, bis-(maltolato)oxovanadium, vanadyl acetylacetonate, or VO(L-Glu(γ)HXM)$_2$ activated lipogenesis to the extent of 4, 5, 16, 30, and 47% of maximal insulin response, respectively, indicating that several *preformed* vanadium(+4) complexes are significantly more potent than free vanadium(+4) in activating lipogenesis.

In Fig. 1B, the same ligands were added to the adipocytic cell suspensions together with vanadium in a 2 to 1 M mixture (rather than preformed vanadium complexes). Under these experimental conditions, picolinate, maltolate, and acetylacetonate did not potentiate the lipogenic activity of $VOCl_2$(+4) and only the mixture of L-Glu(γ)HXM with $VOCl_2$ was as effective as the preformed complex with vanadium(+4) (47 $\pm$ 3% of maximal insulin response). This experimental approach, however, suffers from several drawbacks: differences in stability between complexes at physiological pH; the fact that vanadium and *some* ligands are seemingly unable to associate spontaneously in the external medium (Fig. 1B); and different permeation capabilities of the different ligands into the cell, thus putting into question whether ligands tend to remain in an optimal associated form after permeation. We therefore searched for a more dependable, in vitro technique for ligand classification.

Optimal Vanadium Ligands, When Added Alone to Vanadium-Enriched Adipocytes, Activate Lipogenesis. The basal lipogenic activity of adipocytes enriched with vanadium, obtained by treating rats with vanadate (see *Experimental Procedures*), rose to 15 to 25% of maximal stimulation; maximal insulin response (Fig. 2A) and the dose-response curve to insulin (not shown) were the same in normal and vanadium-enriched adipocytes. This procedure yielded adipocytes that were enriched with vanadium by about 25-fold (see *Experimental Procedures*). In this system, we define an optimal ligand as one that, upon its addition to vanadium-enriched adipocytes by *itself* and at *low concentrations* (10 μM), is capable of permeating into the cell interior. There it associates with the enriched pool of vanadium, independent of whether vanadium is intracellularly bound or free, at (+4) or (+5) state of oxidation, and turns it into an insulinomimetic active species, which thus activates lipogenesis.

Figure 2A shows activation of lipogenesis in vanadium-enriched adipocytes exposed to 10 μM concentration of various ligands. The L-isomers of Glu(γ)HXM and Asp(β)HXM were highly active, reaching levels of 57 and 66%, respectively, of maximal insulin response. L-Ile(α)HXM was less effective (7% maximal insulin response) and all other ligands were inactive.

Figure 2B shows the lipogenic capacity of the same ligands added at 10-fold higher concentrations (100 μM) to control (nonvanadium enriched) rat adipocytes. Again, L-Glu(γ)HXM, L-Asp(β)HXM, and L-Ile(α)HXM[1] activated lipogenesis to 38 $\pm$ 4, 35 $\pm$ 3, and 8.0 $\pm$ 0.7% of maximal insulin response, respectively, whereas none of the other ligands (picolinate, maltolate, citrate, and GSH) had any effect. Similar results were obtained when the enrichment procedure was performed by administering $VOCl_2$(+4) (rather than vanadate) to rats over a period of 4 days before preparing vanadium-enriched rat adipocytes (data not shown).

L-Glu(γ)HXM was found to be unique in its capability to activate lipogenesis in rat adipocytes in the absence of exogenous vanadium. The D-isomer of Glu(γ)HXM, as well as *N*-acetyl-L-Glu(γ)HXM or L-Glu(γ)HXM-α-methyl ester were virtually ineffective (Goldwaser et al., 1999; Fig. 2C).

[1] Essentially the same results were obtained with several other α-amino acid HXMs. For simplicity, L-Ile(α)HXM is referred to throughout the manuscript as a representative of α-amino acid HXMs.

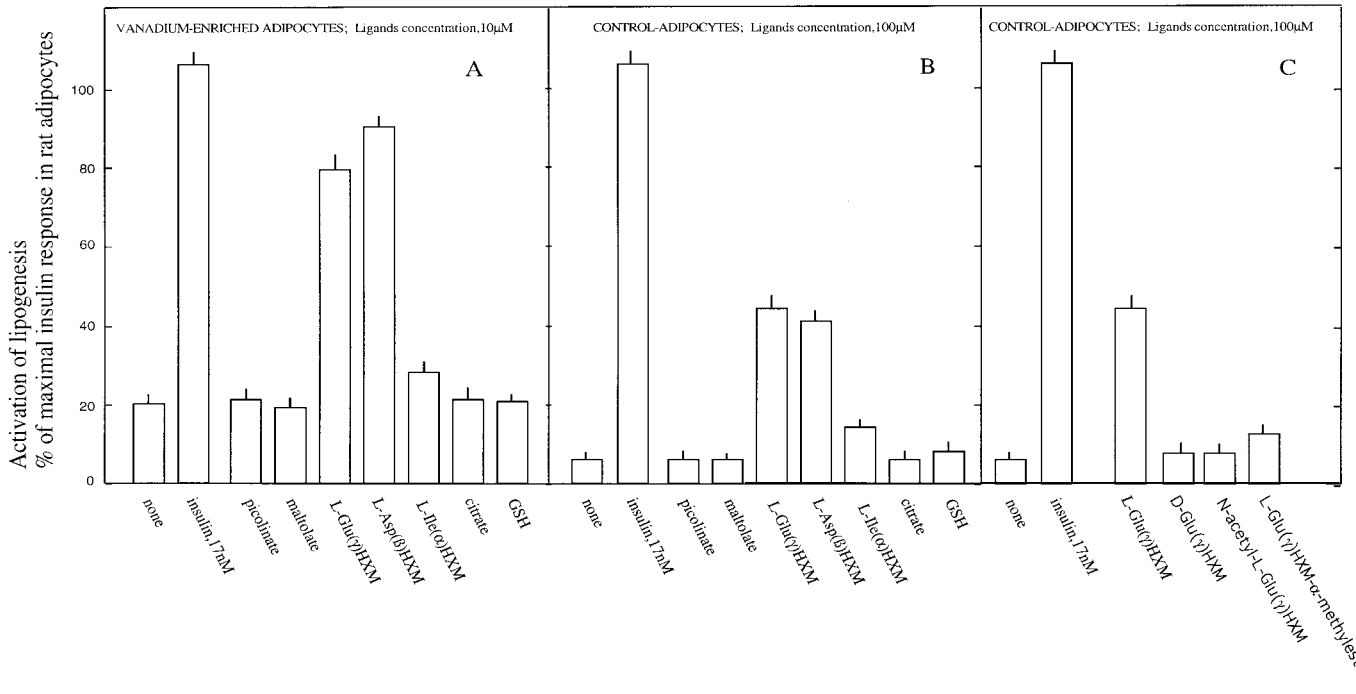

Fig. 2. Capacity of vanadium ligands, in the absence of exogenous vanadium, to activate lipogenesis in vanadium-enriched and in control adipocytes. Male Wistar rats received daily s.c. administrations of $NaVO_3$ for 4 days before the preparation of adipose cells (see under *Experimental Procedures*). A, the vanadium-enriched adipocytes were preincubated with 10 μM concentrations of the ligands listed for 10 min. Lipogenesis was then performed for 2 h. B and C, lipogenesis was performed with control adipocytes and 100 μM concentrations of the ligands.

Establishing Cell-Free Procedures for Evaluating Affinities of Ligands to Vanadium at Physiological pH. The complex aqueous chemistry of vanadium can complicate the interpretation of biological experiments. Gross differences in shape, geometry, and coordinating capacity of the vanadium atom may occur in aqueous media at varying pH values (Butler, 1990). Therefore, we searched for a simple cell-free experimental system for estimating approximate ligand affinities to vanadium(+4) and vanadium(+5) at physiological pH values. The reversal of vanadium-evoked inhibition of acid phosphatase from wheat-germ origin at pH 7.2 was finally found most suitable. This acid phosphatase displays a wide, bell-shaped pH profile (Hollander, 1971). Its K_m value with pNPP is not altered upon elevating the pH from 4.8 to 7.2, whereas the maximum turnover number is decreased by 6- to 7-fold only at the higher pH value (data not shown). Wheat-germ acid phosphatase was documented to be inhibited by vanadate(+5) at a K_i value of 6.7 μM (VanEtten et al., 1974), allowing us to use the low vanadium concentrations, which were effective under physiological conditions. In our assay system, vanadium(+5) is stable. The rate of vanadium(+5) oligomerization is minimal at concentrations lower than 50 μM (Crans, 1994), and reduction to vanadium(+4) does not occur because reductants are absent.

Figure 3 demonstrates the reversal of vanadate(+5)-evoked inhibition of acid-phosphatase, at pH 7.2, at increasing concentrations of ligands of interest. EDTA, picolinate, and maltolate reversed vanadate-evoked acid-phosphatase inhibition with RC_{50} values of 0.08 ± 0.005, 0.8 ± 0.05, and 0.9 ± 0.07 mM, respectively. L-Glu(γ)HXM, L-Asp(β)HXM, and L-Ile(α)HXM showed RC_{50} values of 6.0 ± 0.3, 8.0 ± 0.05, and 8.5 ± 0.7 mM, respectively. Based on the documented affinity of EDTA to vanadate(+5) at neutral pH [$K^{eff} = 1.4 \times 10^4$ M^{-1}, (Crans et al., 1989)], we calculated the apparent formation constants of the ligands with vanadate (Table 1). Picolinate- and maltolate-vanadate complex had a $K^{eff/pH\ 7.2}$ at the range of 1.2 to 1.4×10^3 M^{-1}, whereas the three amino acid hydroxamates showed a considerable lower affinity ($K^{eff/pH\ 7.2} = 1.3$ to 1.9×10^2 M^{-1}); citrate and GSH showed even weaker affinity toward vanadate ($K^{eff/pH\ 7.2} < 10^1$ M^{-1}; Table 1).

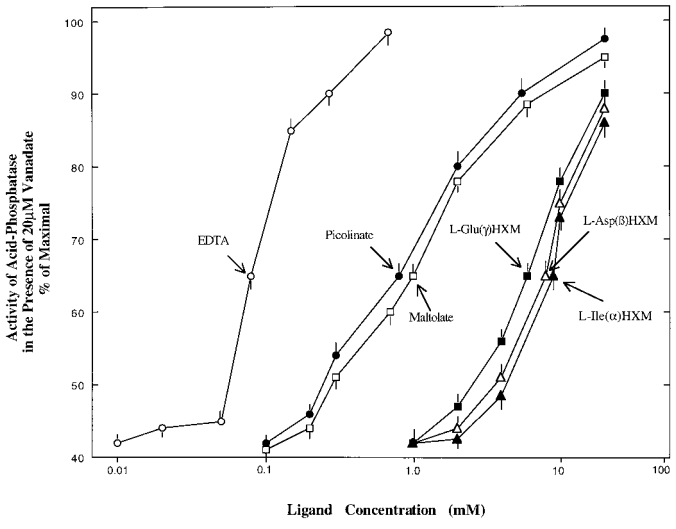

Fig. 3. Reversal of vanadate(+5)-evoked inhibition of acid-phosphatase as a function of ligand concentration at pH 7.2. The assay mixture (0.5 ml) contained 50 mM HEPES, pH 7.2, 1.0 M KCl, 20 μM $NaVO_3$, 0.2 mM pNPP, 100 μg/ml wheat-germ acid-phosphatase, and increasing concentrations of the tested vanadium ligands. Rates of hydrolysis are defined as the quantity of pNPP hydrolyzed during a 10-min incubation period at 25°C and pH 7.2. Rates were determined individually for each ligand concentration in the absence and presence of 20 μM $NaVO_3$.

Thus we assumed that this narrow range of ligand affinities toward vanadium(+5) (namely, $K^{eff/pH\ 7.2} = 1.3$ to 1.9×10^2 M^{-1}; Table 1) is compatible with their high in vitro insulinomimetic potencies. With this assumption, however, an explanation is required for the lower activity of L-Ile(α)HXM, although it associates equally well with vanadium(+5) as L-Glu(γ)HXM and L-Asp(β)HXM do (subsequent paragraph).

Vanadium(+4) Is Preserved as Such at Aqueous Neutral Conditions in the Presence of Weakly Interacting Ligands. Before studying further ligands affinities toward vanadium(+4) at physiological pH, we wished to determine the existence of vanadium(+4), at neutral pH, under the experimental conditions applied here. Previous studies claimed that free vanadium(+4) is stable only at acidic pH (pH <3.0), whereas in aqueous solution at neutral pH it is quickly hydrolyzed and/or slowly oxidized to vanadium(+5) (Macara, 1980). Millimolar concentrations of free vanadium(+4) are rather insoluble at neutral pH values. However, in citrate buffer, a weakly interacting ligand (Crans et al., 1989; and this study) in a solution of up to 10 mM can be maintained in a fully soluble form at pH 7.4. Figure 4, A and B, shows the electron spin resonance (ESR) spectrum of $VOCl_2$, pH <3.0, and in 0.1 M citrate buffer, pH = 7.4. At a neutral pH value, the ESR-spectrum of vanadium(+4) disappeared, but upon acidification at 30, 60, and 90 min after preparation, it reappeared (Fig. 4, C, D, and E). Thus, vanadium(+4) seems to remain in its (+4) oxidation state at aqueous physiological pH. The loss of the ESR signal does not reflect vanadium(+4) hydrolysis, or oxidation, as previously assumed, but rather dimerization, which tends to occur at pH values >3.0 (D. Crans, personal communication).

TABLE 1
Reversal of vanadium (+5)-evoked inhibition of acid phosphatase at pH 7.2 by various chelators

Ligand	Half-Maximal Reversal of Vanadate(+5)-Evoked Inhibition of Acid Phosphatase RC_{50}[a]	$K^{eff/pH\ 7.2}$ Values[b]	Activation of Lipogenesis in Rat Adipocytes
	mM	*[10^3 M^{-1}]*	
EDTA	0.08 ± 0.03	14	−[c]
Picolinate	0.8 ± 0.05	1.4	−
Maltolate	0.9 ± 0.07	1.24	−
L-Glu(γ)HXM	6.0 ± 0.3	0.19	++++[c]
L-Asp(β)HXM	8.0 ± 0.4	0.14	++++
L-Ile(α)HXM	8.3 ± 0.5	0.135	+[c]
L-Arg(α)HXM	8.4 ± 0.7	0.133	+
L-Glu	>140 ± 15	<0.008	−[c]
L-Gln	>170 ± 12	<0.0066	−
L-Asn	>140 ± 12	<0.008	−
Citrate	>200 ± 20	<0.0056	−
GSH	>120 ± 10	<0.0093	−

[a] Performed as described under *Experimental Procedures* and in the legend to Fig. 3.

[b] Calculated based on the documented affinity of EDTA to vanadate at neutral pH under similar experimental conditions (Crans et al., 1989).

[c] (−), (+), and (++++) refer to ligands that are inactive, partially active, or highly active, respectively, in activating lipogenesis when added at 100 μM concentration to control adipocytes or at 10 μM concentration to vanadium-enriched adipocytes.

To further confirm vanadium(+4) stability in a more 'natural' experimental system, the procedure of Wilsky et al. (1984) with *S. cerevisiae* was adapted, using higher pH values. In this eukaryotic cell-line, vanadium(+5), but not vanadium(+4), effectively inhibits H^+ ATPase, and therefore arrests cell growth (Wilsky et al., 1984). In Fig. 5, suspensions of *S. cerevisiae* were incubated at pH 7.4, 37°C, with the indicated concentrations of vanadate or vanadyl for 5 h before evaluating cell growth (see *Experimental Procedures*). At 2.5 and 5.0 mM vanadate(+5), cell division was arrested by 62 and 100%, respectively. In contrast, the same concentrations of vanadyl(+4) had no inhibitory effect on cell growth, indicating none or negligible conversion of vanadium(+4) to vanadium(+5) at physiological pH and temperature.

Ligand Affinities toward Vanadium(+4). Fig. 6 shows the reversal of $VOCl_2$(+4)-evoked inhibition of acid phosphatase by EDTA and other ligands at pH 7.2. Half-maximal values taken from Fig. 6 are 27 ± 3, 170 ± 14, and 480 ± 30 μM for EDTA, picolinate, and maltolate, respectively. RC_{50} values for the amino acid hydroxamates were 6.0 ± 0.4 and 21 ± 3 mM for L-Glu(γ)HXM and L-Ile(α)HXM, respectively. Reduced glutathione, citrate, L-glutamine, L-asparagine, and L-glutamic acid at 100 mM concentration did not reverse vanadium(+4)-evoked inhibition of acid-phosphatase (summarized in Table 2). Thus, under similar experimental conditions, EDTA is 3-fold more potent in reversing vanadium(+4)- than vanadium(+5)-evoked inhibition of acid phosphatase. Table 2 presents the ligands' affinities for vanadium(+4), taking EDTA as an internal standard and using $K^{eff/pH\ 7.2}$ of $4.14 \times 10^4\ M^{-1}$. Affinities of formations were 5.8 ± 0.3 and 2.07 ± 0.2 × $10^3\ M^{-1}$ for picolinate and maltolate, repectively, and 1.65 ± 0.2, 1.84 ± 0.3, and 0.47 ± 0.03 × $10^2\ M^{-1}$ for L-Glu(γ)HXM, L-Asp(β)HXM, and L-Ile(α)HXM, respectively. Thus, at pH 7.2, the affinity of L-Ile(α)HXM for vanadium(+4) is considerably weaker than for the vanadium(+5) anion (Tables 1 and 2). This, however, is not the case with either L-Glu(γ)HXM or L-Asp(β)HXM, which have nearly the same binding affinities toward both vanadium(+4) and (+5) at physiological pH (Table 3).

Spectroscopic Monitoring of Insulinomimetic-Potentiating Vanadium Ligands. Vanadium ions in aqueous media assume several topographies, depending on the type and number of ancillary ligands occupying the coordination sphere of the vanadium atom (reviewed in Butler, 1990). Here we determined whether these shape-differences are reflected by measurable spectroscopic changes. In Fig. 7, $VOCl_2$ (2 mM, +4) maintained at 0.1 M acetic acid, pH 2.7, was titrated with various ligands, and the absorbance at 300 nm was recorded. After preliminary studies, pH 2.7 to 3.0 was chosen for conducting these spectroscopic studies, because vanadium(+4) itself absorbed negligibly at this wavelength ($\epsilon_{300\ nm}$ = 50 ± 3).

Absorption at 300 nm was increased from 0.1 to 0.32, 0.43, 0.55, and 0.72 upon titrating $VOCl_2$ with 1, 2, 3, and 5 M equivalents, respectively, of L-Glu(γ)HXM (Fig. 7A); further addition of L-Glu(γ)HXM (up to 10 M excess) did not significantly increase the absorbance. A similar pattern was obtained upon titrating $VOCl_2$ with L-Asp(β)HXM (Fig. 7B). Addition of 1 equivalent of L-Ile(α)HXM increased the absorbance from 0.1 to 0.27 (Fig. 7C), but additional L-Ile(α)HXM, up to 10 equivalents, did not increase absorbance any further. One equivalent EDTA, which forms a 1:1 tight complex with $VOCl_2$ (Przybrowsky et al., 1965), increased the absorbance from 0.1 to 0.27; higher concentrations of EDTA had no further effect (Fig. 7D). GSH, Asn, Glu, and Gln had no effect even at 10 M excess (Fig. 7E). None of the ligands presented in the figure absorb, by itself at 300 nm.

Figure 8 demonstrates spectroscopic titrations of $VOCl_2$(+4) with picolinate, maltolate, and acetylacetonate. In these experiments, we used 10-fold lower concentrations of $VOCl_2$ (0.2 mM), becuase these ligands interact about 10-fold more strongly with vanadium(+4) (Table 2). Also, the increase in absorbance was monitored at 324 nm, because maltolate and picolinate by themselves show some absorbance at 300 nm but not at 324 nm (not shown).

Titration of $VOCl_2$ (0.2 mM, in 0.1 M acetic acid, A_{324} = 0.007) with 1, 2, 3, 5, and 10 equivalents of maltolate elevated the absorbance at 324 nm to 0.17, 0.35, 0.53, 0.65, and 0.75, respectively; similar concentrations of acetylacetonate elevated absorbance to 0.1, 0.25, 0.38, 0.49, and 0.56, respectively. Titration of $VOCl_2$ with picolinate did not increase absorbance (Fig. 8).

In Fig. 9, $VOCl_2$ was titrated with L-Glu(γ)HXM, *N*-acetyl-L-Glu(γ)HXM, and L-Glu(γ)HXM-methylester. *N*-Acetyl-L-Glu(γ)HXM was indistinguishable from L-Glu(γ)HXM in increasing the absorbance at 300 nm. In contrast, L-Glu(γ)HXM-methyl ester (1 to 3 equivalents) was ineffective.

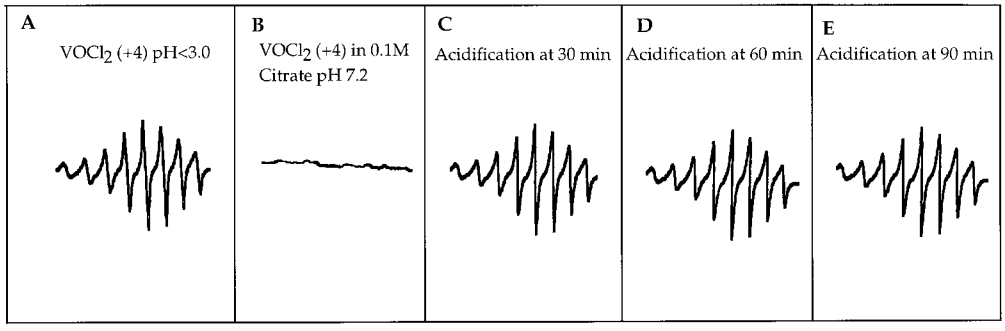

Fig. 4. ESR spectra of vanadium(+4). Loss of the typical ESR spectrum of vanadium(+4) in aqueous buffer at pH 7.4 and its reappearance upon acidification at different time points. ESR spectrum of 10 mM vanadium dichloride at pH <3.0 (A). B, as in A, but in 0.1 M citrate buffer, pH 7.2. C, as in A, but in 0.1 M citrate-buffer, pH 7.2, maintained for 30 min at 25°C and then acidified with concentrated HCl to pH <3.0. D, as in B, but maintained at pH 7.2, 25°C for 60 min before acidification. E, as in B, but maintained in citrate buffer, pH 7.4, for 90 min before acidified and remonitored. A Bruker ESR-spectrometer (ER-200) was used. Recording conditions were as follows: modulation amplitude, 3.2G; microwave power, 24 mW; time constant, 640 ms; scan time, 200 sec; sweep width, 2000G; and center field, 3500G.

Discussion

Complexes of vanadium with L-Glu(γ)HXM (Goldwaser et al., 1999) and with L-Asp(β)HXM (this study) are especially potent in activating glucose uptake and glucose metabolism in rat adipocytes. In STZ-rats, L-Glu(γ)HXM·vanadium (2:1) is 4 to 5 times more powerful than free vanadium in reducing circulating glucose levels (Goldwaser et al., 1999). In general, for a ligand and vanadium to act synergistically, particularly in vivo, certain requirements must be fulfilled. Most crucial is the appropriate kinetics of complexation and decomplexation, which ensures delivery and adequate presentation of the metalo-oxide ion in an associated form to key sites of glucose metabolism (Orvig et al., 1995; Setyawati et al., 1998). Our notion of L-Glu(γ)HXM being an optimal vanadium ligand is based on its high efficacy in STZ rats in vivo, and in several type II diabetic rodents (Goldwaser et al., 1999; I. Goldwaser, M. Fridkin, and Y. Shechter, manuscript in preparation).

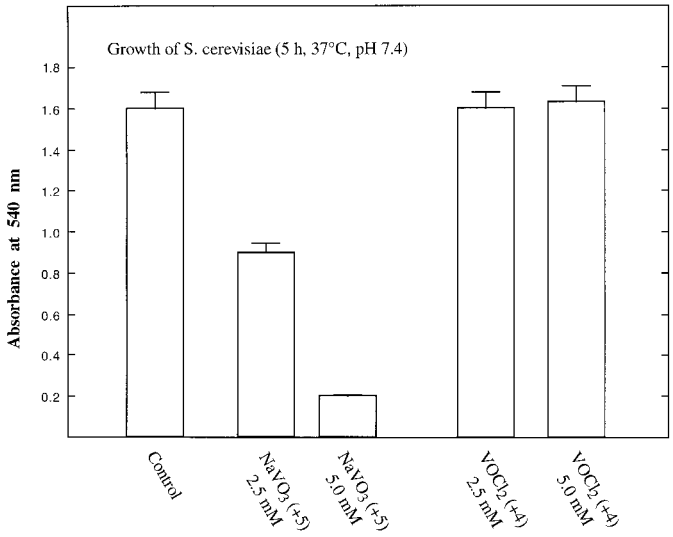

Fig. 5. Growth response of *S. cerevisiae* in the presence of vanadate and vanadyl. Cells were grown overnight and resuspended in yeast extract/peptone/dextrose medium, pH 7.4. The suspension was divided into several vials and incubated for 5 h at 37°C in the presence or absence of $NaVO_3$(+5) or $VOCl_2$(+4). Cell growth was evaluated spectroscopically at 540 nm.

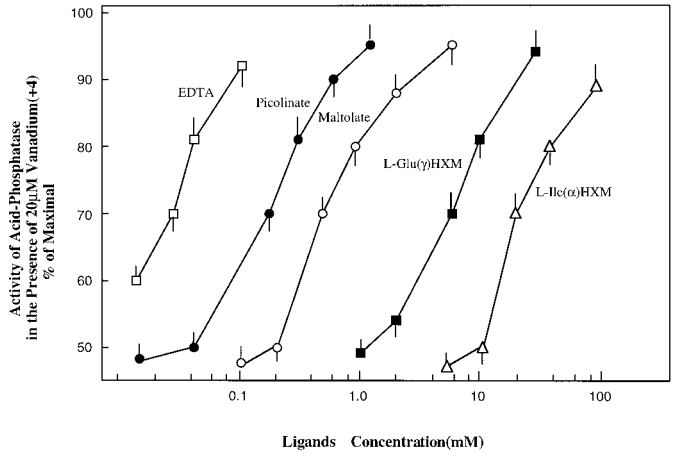

Fig. 6. Reversal of vanadium(+4) evoked inhibition of acid phosphatase as a function of ligand concentration at pH 7.2. The assay mixture (0.5 ml) contained 50 mM HEPES, pH 7.2, 1.0 M KCl, 20 μM $VOCl_2$(+4), 0.2 mM pNPP, and increasing concentrations of the tested vanadium ligands. Rates of hydrolysis are defined as the quantity of pNPP hydrolyzed during a 10-min incubation period at 25° and pH 7.2. Rates were determined individually for each ligand concentration in the absence and presence of 20 μM $VOCl_2$.

This study demonstrated that L-Glu(γ)HXM and L-Asp(β)HXM at 10 μM concentrations activated lipogenesis in vanadium-enriched and nonenriched rat adipocytes (Fig. 2). The α-amino acid hydroxamates (represented by L-Ile(α)HXM) were about 7 times less potent. Picolinate and maltolate, although their complexes with vanadium(+4) in STZ-rats were somewhat more active than free vanadium, did not stimulate vanadium-enriched adipocytes. Examining the affinities of formation of all these ligands toward vanadium(+5), we found that the amino acid HXMs have calculated $K^{eff/pH\ 7.2}$ values significantly lower than picolinate and maltolate. We assumed that this narrow and rather low range of amino acid HXM ligand affinities, toward vanadium(+5) ($1.3–1.9 \times 10^2\ M^{-1}$) coincide with the potentiating effect of vanadium upon complexation; thus, we searched further for an explanation for the lower synergistic potencies of the α-amino acid hydroxamates.

With the recognition that vanadium(+4), although paramagnetically silent at pH 7.2, remains in its +4 state of oxidation (Fig. 4 and 5), we studied ligands' affinity for vanadyl(+4). We obtained $K^{eff/pH\ 7.2}$ values of 1.65 and $1.85 \times 10^2\ M^{-1}$ for L-Glu(γ)HXM and L-Asp(β)HXM, respectively, whereas the $K^{eff/pH\ 7.2}$ for L-Ile(α)HXM was considerably lower ($0.47 \times 10^2\ M^{-1}$; Table 2).

Comparison of ligands' affinities for vanadium(+4) and (+5) revealed higher affinities of maltolate (4.2-fold) and picolinate (1.7-fold) toward vanadium(+4) compared with vanadium(+5). L-Glu(γ)HXM and L-Asp(β)HXM, however, showed nearly the same affinity toward vanadium(+4) and vanadium(+5) (affinity ratios of 0.9 and 1.3, respectively; Table 3). L-Ile(α)HXM is an exception: its affinity for vanadium(+5) exceeded by nearly 3-fold its affinity for vanadium(+4) (Table 3). We therefore concluded that comparable affinities of a ligand toward both states of vanadium oxida-

TABLE 2
Reversal of vanadium(+4)-evoked inhibition of acid phosphatase at pH 7.2 by various chelators

Ligand	Concentration for Half-Maximally Reversing Vanadium(+4)-Evoked Inhibition of Acid Phosphatase	Formation Constant $K^{eff/pH\ 7.2}$
	mM	*[$10^3\ M^{-1}$]*
EDTA	0.024 ± 0.003	41.4
Picolinate	0.17 ± 0.008	5.8
Maltolate	0.48 ± 0.03	2.07
L-Glu(γ)HXM	6.0 ± 0.4	0.165
L-Asp(β)HXM	5.4 ± 0.3	0.184
L-Ile(α)HXM	21 ± 3	0.047
GSH	>100	<0.01
Citrate	>100	<0.01
L-Gln	>100	<0.01
L-Asn	>100	<0.01
L-Glu	>100	<100

tion are beneficial for optimal synergistic efficacy. Figure 2C suggests that nonmodified α-amino, α-carboxyl, and the L configuration are required for Glu(γ)HXM entry into the adipose cell. The free α-carboxyl moiety seems necessary for associating with the vanadyl(+4) cation but not with the vanadate(+5) anion, as also suggested, inferred from the spectroscopic measurements (Fig. 9).

Having established the K_d value for L-Glu(γ)HXM·vanadate (2:1), we can now estimate the complex circulating levels shortly after i.p. administration to rats under the experimental

TABLE 3
Affinity of various studied ligands toward the vanadyl(+4) cation and the vanadate(+5) anion at physiological pH

Ligand	$K^{eff/pH\ 7.2}$ toward Vanadium(+5)	$K^{eff/pH\ 7.2}$ toward Vanadium(+4)	Vanadium(+4)/ Vanadium(+5) Affinity Ratio	Ligands Bound to mol Vanadium(+4) (pH 2.7)
				mol
EDTA	$1.4 \times 10^4\ M^{-1}$	$4.1 \times 10^4\ M^{-1}$	2.93	1.0
Picolinate	$1.4 \times 10^3\ M^{-1}$	$5.8 \times 10^3\ M^{-1}$	4.14	N.D.
Maltolate	$1.24 \times 10^3\ M^{-1}$	$2.07 \times 10^3\ M^{-1}$	1.67	3–4
L-Glu(γ)HXM	$1.9 \times 10^2\ M^{-1}$	$1.65 \times 10^2\ M^{-1}$	0.87	3–4
L-Asp(β)HXM	$1.4 \times 10^2\ M^{-1}$	$1.84 \times 10^2\ M^{-1}$	1.31	3–4
L-Ile(α)XHM	$1.35 \times 10^2\ M^{-1}$	$0.47 \times 10^2\ M^{-1}$	0.35	1.0

N.D., not determined.

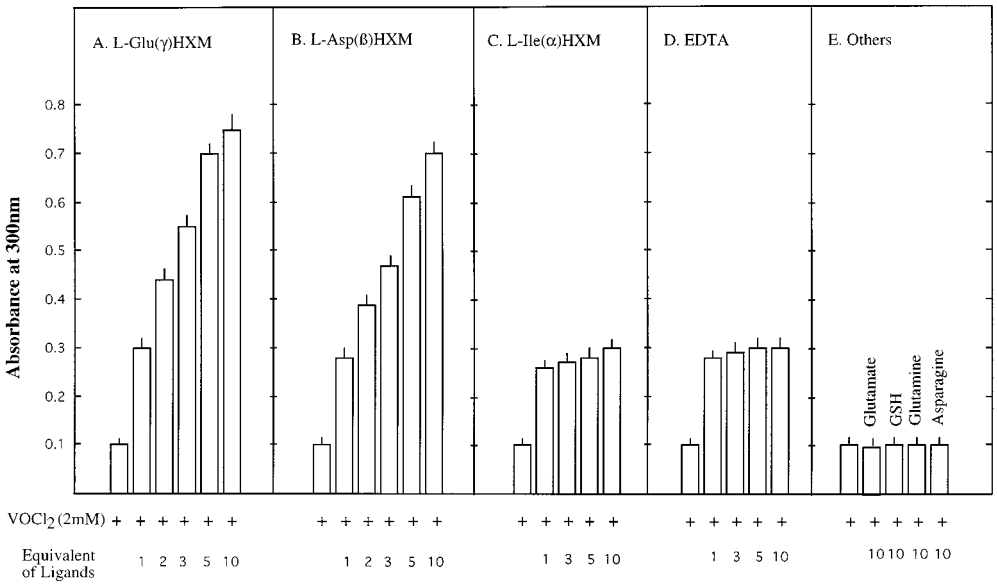

Fig. 7. Spectroscopic titration of vanadium(+4) with ligands at 300 nm. A solution of $VOCl_2$ (0.3 ml, 2 mM in 0.1 M acetic acid) was placed in a cuvette and the absorbance at 300 nm was monitored. This was sequentially titrated with 1 to 10 equivalents of each of the ligands and the absorbance was measured after each addition. Each equivalent was 3 μl from a 200 mM ligand solution (in 0.1 M acetic acid). None of the vanadium ligands presented in the figure absorb at 300 nm.

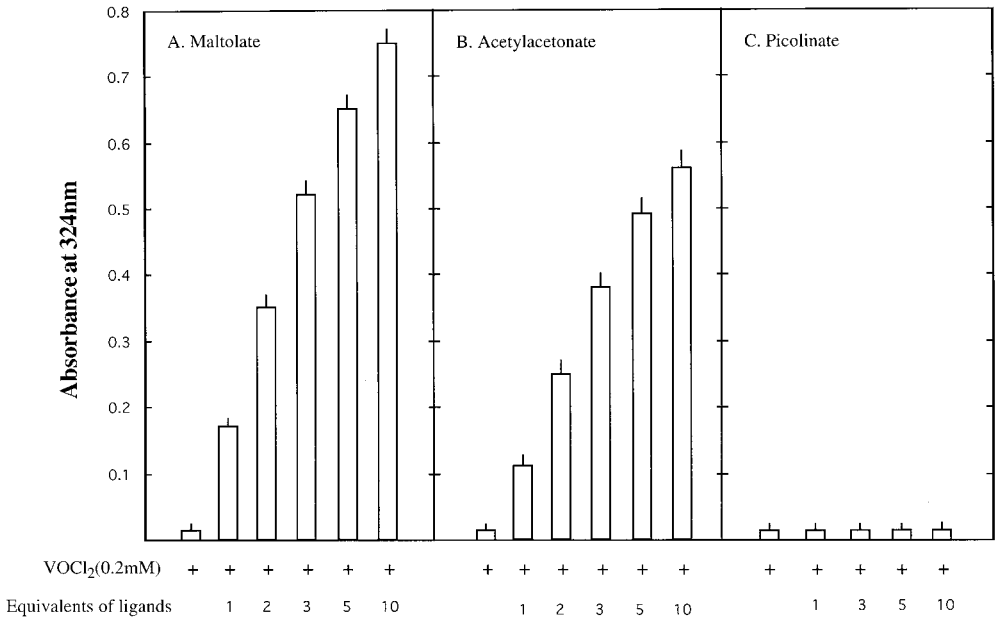

Fig. 8. Spectroscopic titration of vanadium(+4) with maltolate, acetylacetonate, and picolinate at 324 nm. Spectroscopic titration was performed essentially as described in the legend to Fig. 7, except that a solution of 0.2 mM $VOCl_2$ was titrated with the studied ligands, and absorbance was monitored at 324 nm. None of the individual ligands presented in the figure absorb at 324 nm.

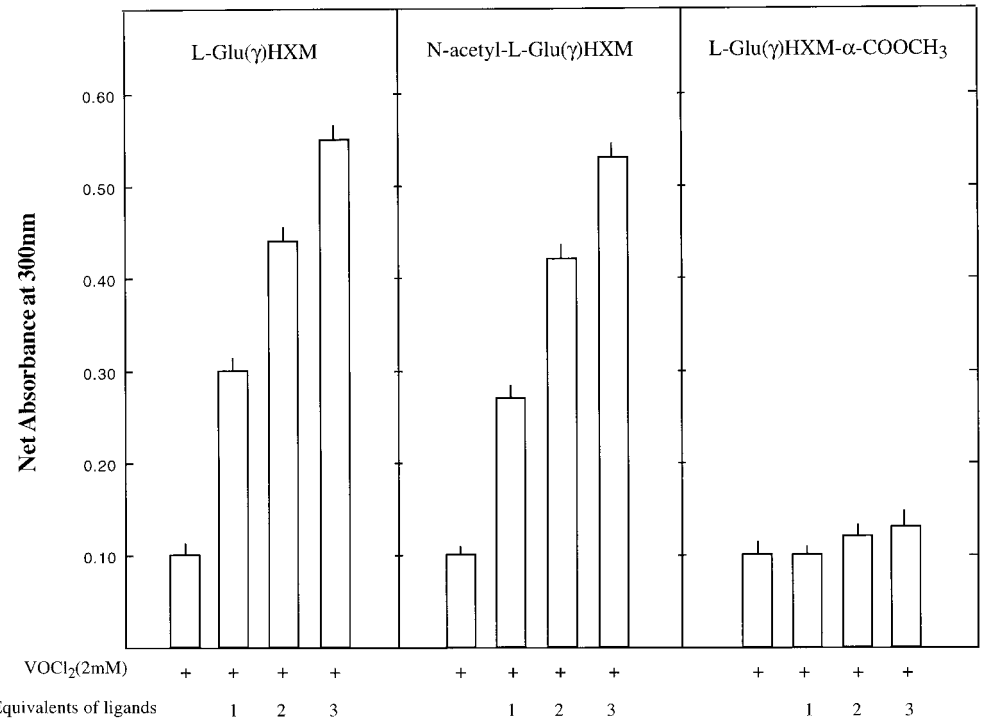

Fig. 9. Spectroscopic titration of $VOCl_2$ with chemically modified derivatives of L-Glu(γ)HXM: *N*-acetyl-L-Glu(γ)HXM, but not L-Glu(γ)HXM-$COOCH_3$, associates with vanadium(+4). $VOCl_2$ (2.0 mM) was titrated with L-Glu(γ)HXM and its derivatives. The absorbance at 300 nm was monitored as described in the legend to Fig. 7. L-Glu(γ)HXM-$COOCH_3$ has some absorbance at 300 nm, which was subtracted to obtain net absorbance at this wavelength.

conditions applied in our previous study. Considering that the amount of complex administered was 20 μmol/rat and assuming a 10-fold dilution, the complex level is estimated at 2 μmol/ml, of which about 20% would be complexed at physiological pH, using a K_d value of $6.25 \pm 1.0 \times 10^{-3}$ M. This level drops within several hours after administration. Nevertheless, normoglycemia has been attained even at 24 h after administration (Fig. 5 in Goldwaser et al., 1999). Thus an initial complex-circulating level of about 0.4 μmol/ml is sufficient for maintaining long-lasting normoglycemic state.

Finally, simple spectroscopic titration of vanadium(+4) with 1, 2, 3, 5, and 10 equivalents of insulinomimetic-active ligands revealed large graded increases in the absorbency of vanadium(+4) at 300 nm with L-Glu(γ)HXM, or L-Asp(β)HXM, but not with L-Ile(α)HXM, indicating the efficacy of optimal ligands in inducing intense 'spectroscopic-signature' upon associating with vanadium(+4) at slightly acidic, aqueous media. Although these spectroscopic measurements could not be carried out at physiological pH, comparison of the patterns obtained for the different ligands at pH 2.7 to 3.0 may turn out to be useful, as a means of predicting properties of complexes at physiological pH. Thus ligands forming high ultraviolet absorbing complexes also 'force' vanadium into an insulinomimetically-active form. The α-amino acid HXMs cannot induce this desirable structural configuration. Maltolate and acetylacetonate also form intense ultraviolet absorbing complexes with vanadium(+4) (Fig. 8), but they differ from L-Glu(γ)HXM in forming a much tighter complex with vanadium and in possessing higher affinity for vanadium(+4) than for vanadium(+5) (Tables 1 to 3). These ligands are, therefore, weaker, less effective vanadium donators.

In summary, we propose that optimal insulinomimetic synergizing ligands have to fulfill these three criteria: 1) they must complex with vanadium(+5) within the narrow range of $K^{eff/pH\ 7.2} = 1.3$ to $1.9 \times 10^2\ M^{-1}$ at physiological pH value; 2) they must have nearly equal binding affinities for vanadium(+4) and vanadium(+5) at this pH range; and 3) they must form, with vanadium(+4), an intense ultraviolet-absorbing complex upon titrating the metalo-oxide with 1 to 3 M equivalents of selected ligands at pH ~3.0. Assuming a 2:1 M stoichiometry-dimeric type of complex, each mole of an optimal ligand contributes with a $\Delta\epsilon_{324}=1680 \pm 100$ upon association with vanadium(+4) (calculated from Fig. 8). This is an extensive, large signal, at a wavelength at which the individual components of the complex absorb negligibly or not at all.

Finally, a free α-carboxyl moiety (e.g., as in L-Glu(γ)HXM) seems necessary for ligand-vanadyl(+4) cation binding, whereas the α-amino side chain is not (Table 3; Fig. 9). In newly designed second generation amino acid HXMs, substitutions can therefore be potentially introduced through the α-amino moiety that augment the insulinomimetic effects of vanadium. This and other aspects raised here are currently being studied.

Acknowledgments

We thank Elana Friedman for typing the manuscript and Dr. Sandra Moshonov and Yigal Avivi for editing it.

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