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Mn^{2+}-binding properties of a recombinant protein-tyrosine kinase derived from the human insulin receptor

(conformational change/secondary structure/electron paramagnetic resonance/circular dichroism)

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ABSTRACT **The divalent cation-binding properties of the human insulin receptor tyrosine kinase domain were examined kinetically and by electron paramagnetic resonance and circular dichroic spectroscopy. The protein-tyrosine kinase activity of the purified cytoplasmic domain can be activated nearly 10-fold by 3 mM Mn^{2+} in the presence or absence of 5 mM Mg^{2+}. Electron paramagnetic resonance spectra of the purified, acid-denatured kinase domain and assays of EDTA-treated kinase show that the purified protein does not possess residual, tightly bound Mn^{2+}. Electron paramagnetic resonance spectroscopy was used to directly measure the binding constant of the kinase domain for Mn^{2+}. The results indicate that the recombinant cytoplasmic domain of the human insulin receptor does not bind Mn^{2+} tightly in the absence or presence of MgATP ($K_d > 0.8$ mM). Furthermore, the enzyme does not show a strong preference for MnATP binding when both MgATP and MnATP are present. The far-ultraviolet circular dichroic spectrum of this domain is characterized by a negative maximum at 207 nm. In the presence of Mn^{2+}, but not Mg^{2+}, changes in the mean residue-weight ellipticity at 207 nm occur that are consistent with a decrease in α-helical content. The addition of ATP to Mn^{2+}-bound protein does not further perturb the spectrum. We conclude that Mn^{2+} ions, although they bind weakly, induce an activating conformational change in the secondary structure of the human insulin receptor cytoplasmic domain. Activation by Mn^{2+} is unlikely to be significant in intact cells, but it may mimic the action of a physiological activator.**

The human insulin receptor's tyrosine kinase activity is essential for the mediation of insulin action in cells (reviewed in ref. 1). This was most clearly shown by the analysis of a variety of point mutations in the conserved ATP-binding site of the cytoplasmic domain (2–4). For example, the replacement of Lys-1018 with alanine eliminated the tyrosine kinase activity and coincidentally abrogated a number of insulin-dependent physiological effects (1–5). Thus, insight into the regulation of this kinase activity is critical for a further understanding of insulin action.

As a class, the growth factor receptor protein-tyrosine kinases are stimulated by Mn^{2+} in the presence or absence of Mg^{2+}. This is not true for all protein kinases, notably the serine/threonine kinases (for example, the type II bovine heart, cAMP-dependent protein kinase requires divalent cations for activity yet Mg^{2+} satisfies this requisite better than Mn^{2+}; ref. 6). This unique activation of tyrosine kinases by Mn^{2+} warrants further study. In particular, the strong preference of the human insulin receptor protein kinase activity for Mn^{2+} has suggested a functional and/or structural role for this divalent cation (7–11).

Several kinetic studies have addressed the activation mechanism of the insulin receptor tyrosine kinase by Mn^{2+}. It has been concluded by one group (12) that the divalent cation complexes with both ATP and a separate, distinct site in the kinase. Furthermore, Mn^{2+} binding to the kinase may increase the enzyme's affinity for MnATP. Nemenoff *et al.* (13) have proposed that the insulin receptor tyrosine kinase may have a greater affinity for MnATP than for MgATP as evidenced by the ability of Mg^{2+} to activate to a greater extent than Mn^{2+} at high ATP concentrations. These proposed scenarios for the mechanism of Mn^{2+} action are intriguing, but several important questions remain unanswered. The binding constants of the human insulin receptor protein-tyrosine kinase domain for Mn^{2+} and MnATP have not been measured by direct physicochemical methods; nor have any structural perturbations induced in the kinase domain by divalent cations been reported or characterized.

The human insulin receptor protein-tyrosine kinase domain has been successfully overproduced and purified from a baculovirus/insect cell system (R-BIRK, a recombinant protein that includes residues 941–1343 of the human insulin receptor; ref. 14). The availability of milligram quantities of virtually homogeneous R-BIRK has set the framework for conducting these physicochemical studies addressing the mechanism of Mn^{2+} action. Direct measurement of Mn^{2+} concentration in solution can be conveniently made by electron paramagnetic resonance (EPR). When bound either to ATP or to protein, the intensity of the Mn^{2+} absorption by EPR decreases substantially. This permits direct binding studies of insulin receptor kinase with Mn^{2+} or MnATP and permits analysis for tightly bound Mn^{2+}.

Villalba *et al.* (15) have characterized the near- and far-ultraviolet circular dichroic (CD) spectra for the native R-BIRK. In the investigations presented here, global conformational changes induced by Mn^{2+} binding to R-BIRK have been observed by monitoring perturbations in the CD spectra. These measurements provide an independent determination of the Mn^{2+}–insulin receptor kinase binding constant as well as reporting on the resulting structural changes in the protein.

Most importantly, to function as an activator in intact cells, Mn^{2+} must be capable of interacting with the insulin receptor tyrosine kinase under physiological conditions. Mn^{2+} could be tightly bound to the protein at all times, or it could activate with a binding constant near the physiological concentration of the ion. The liver content of total manganese is near 35 μM (based on cell water content); however, free Mn^{2+} is less than 1 μM (16). With intracellular Mg^{2+} in the millimolar range,

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Abbreviation: R-BIRK, baculovirus insulin receptor kinase that includes residues 941–1343 of the human insulin receptor.

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Mn^{2+} would compete poorly for ATP to form MnATP. However, as suggested above (13, 14), it is possible that the insulin receptor tyrosine kinase binds MnATP much tighter than MgATP, thereby accounting for the observed activation by Mn^{2+}. The experiments described in this work directly test each of the mechanisms that could explain the reported activation of receptor tyrosine kinases by Mn^{2+}.

MATERIALS AND METHODS

Materials. Histone H2b was purchased from Boehringer Mannheim. [γ-^{32}P]ATP was from Amersham (3000 Ci/mmol; 1 Ci = 37 GBq). Poly(Lys) (average molecular mass 17 kDa) and $MgCl_2$ were from Sigma. $MnCl_2$ was obtained from Fisher, and Tris (ultra-pure) was from Schwarz/Mann. The expression and purification of R-BIRK were performed as described (15). Studies were conducted with multiple independent preparations that yielded R-BIRK of greater than 95% homogeneity with a specific activity for histone of about 70 nmol/min per mg.

Protein Kinase Assay. R-BIRK (17 ng) was assayed with 12.5 μg of histone H2b in a 30 μl volume at 30°C for 15 min. The basic reaction mixture contained 50 mM Tris/HCl buffer (pH 7.4 at room temperature), 1 mM dithiothreitol, bovine serum albumin at 1 mg/ml, and 100 μM [γ-^{32}P]ATP (10 cpm/fmol). Reactions were terminated by adding Laemmli sample buffer (17)/20 mM EDTA/10 mM dithiothreitol. Samples were boiled for 5 min and loaded onto $NaDodSO_4$/17% polyacrylamide gels. Quantitation was achieved by cutting out the phosphoproteins, after staining and drying the polyacrylamide gel, and measuring the radioactivity in the slices by scintillation counting.

EPR Spectroscopy. Mn^{2+} concentrations were quantitated by the relative extent of microwave absorbance in an EPR spectrometer. Sample size was 30 μl, contained in small-diameter quartz capillary tubes. The instrument parameters were as follows: scan range, 2000 gauss; center field set at 3350 gauss; time constant, 0.5 sec; scan time, 16 min; modulation amplitude, 5 gauss; modulation frequency, 100 kHz; receiver gain, 5×10^4; microwave power, 20 mW; and microwave frequency, 9.522 GHz. The spectra were taken at room temperature.

CD Spectroscopy. The R-BIRK spectra were measured in 50 mM Tris/HCl buffer (pH 7.4 at room temperature) with 100 mM NaCl (and with the ligand additions indicated), on an Aviv-modified Cary 60 spectropolarimeter. Spectra were recorded at a scanning speed of 0.08 nm/sec. The optical path of the cell for far-ultraviolet CD spectroscopy was 0.1 cm. The results are expressed as mean residue-weight ellipticities ($[\theta']$) in units of degree·cm^2·dmol^{-1}. Values were calculated on the basis of 118 as mean residue-weight in R-BIRK.

Fluorescence Studies. R-BIRK (20 ng/ml) in 50 mM Tris/HCl buffer (pH 7.4 at room temperature) containing 100 mM NaCl was monitored for intrinsic fluorescence perturbations of emission spectra (excitation wavelength, 295 nm) in the presence of poly(Lys) (2–10 μg/ml), 4 mM Mn^{2+}, or 4 mM Mg^{2+}. Spectroscopic measurements were conducted with a Perkin–Elmer MPF-44B spectrofluorometer operated in the ratio mode. Spectra were recorded at 20°C in cells with an optical path of 1.0 cm, at a scanning speed of 1 nm/sec.

RESULTS

Response of R-BIRK Activity to Mn^{2+} and Mg^{2+}. The effect of increasing the concentration of divalent cations on R-BIRK tyrosine kinase activity was investigated. When assays were conducted in the presence of 100 μM ATP and 5 mM Mg^{2+}, a 10-fold activation of R-BIRK kinase activity was observed with Mn^{2+} concentrations greater than 1 mM (Fig. 1). Mg^{2+} concentrations up to 25 mM did not activate. The activating effect of Mn^{2+} is apparently not dependent upon the exogenous substrate, since it has been reported that the phosphorylation of microtubule-associated proteins and tubulin is stimulated to the same extent as that of histone under similar conditions (18). Furthermore, autophosphorylation also exhibits a Mn^{2+}-dependent activation (2- to 5-fold), which cannot be compensated for by Mg^{2+}.

Increasing the ATP concentration from 100 μM to 1 mM did not abolish the ability of Mn^{2+} to activate R-BIRK, as has also been reported for the holoreceptor (13). Likewise, the incorporation of phosphate into histone that is catalyzed by R-BIRK in the presence of Mg^{2+} alone (10 mM) increased 17-fold when the ATP concentration rose from 100 μM to 1 mM (data not shown). Overall, the *in vitro* activity of R-BIRK requires Mn^{2+} in the same manner as has been reported for the human insulin receptor *in vitro* (7–11, 13, 14). The concentration of Mn^{2+} that resulted in half-maximal activation was estimated to be 0.9 mM from conversion of the data in Fig. 1*B* to a Lineweaver–Burk plot. Preincubation of R-BIRK with EDTA (up to 100 μM) did not detectably decrease tyrosine kinase activity (Fig. 1). Thus the low basal activity of R-BIRK as purified from the baculovirus/insect cell system is not a result of traces of other divalent cations or of EDTA-sensitive bound metal ions.

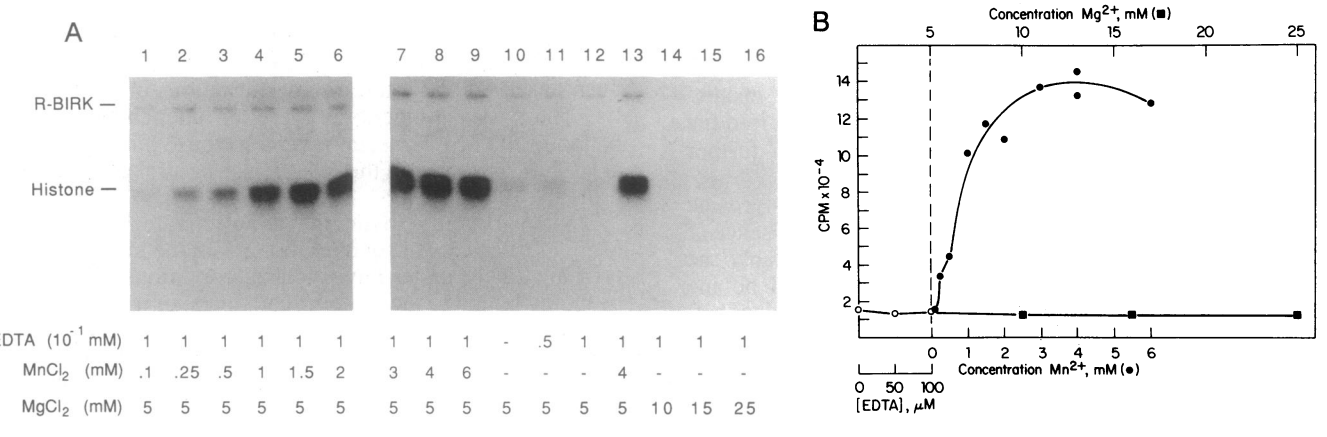

FIG. 1. Activation of R-BIRK by Mn^{2+}. (*A*) Electrophoretic analysis of histone kinase reactions. Assays were conducted with purified R-BIRK as described with the indicated additions of EDTA, $MnCl_2$, and $MgCl_2$. The phosphoproteins were separated by electrophoresis in a $NaDodSO_4$/17% polyacrylamide gel, which was subsequently fixed, stained, dried, and subjected to autoradiography. (*B*) Graphical representation of the activation of R-BIRK by Mn^{2+}. The gel represented in *A* was dried and the ^{32}P incorporated into the histone band was measured by scintillation counting of dried gel slices. Lanes 1–9 and 13, effect of Mn^{2+} (●); lanes 10–12, effect of EDTA (○); lanes 14–16, effect of Mg^{2+} (■).

Mn^{2+} Content of Purified R-BIRK. The Mn^{2+} content of purified R-BIRK was determined by denaturing the protein with perchloric acid and analyzing the extract by EPR. Conditions were selected that could have easily detected a stoichiometry of 0.1 Mn^{2+} ion per R-BIRK monomer. Thus, the analysis could establish whether the Mn^{2+}-independent activity (see Fig. 1*B*) was due to a residual content of tightly bound, EDTA-insensitive Mn^{2+}. Fig. 2 shows that R-BIRK contains less than 0.03 Mn^{2+} ion per protein subunit. Thus, the Mn^{2+}-independent activity of R-BIRK is not due to residual bound Mn^{2+} and the protein as isolated is not a manganometalloprotein.

Estimation of the R-BIRK Mn^{2+} and MnATP Binding Constants. The activation by Mn^{2+} in Fig. 1*B* is complicated by the presence of free Mn^{2+}, free Mg^{2+}, MgATP, and MnATP in solution. The activation constant of 0.9 mM is therefore an apparent constant. When Mn^{2+} is bound to protein, the EPR signal decreases, usually by a factor of approximately 100 (19). Thus the signal of free Mn^{2+} can be used to monitor protein–Mn^{2+} binding. The free Mn^{2+} content of a solution containing 88 μM R-BIRK and 59 μM Mn^{2+} showed no significant decrease in free Mn^{2+} concentration under experimental conditions that could have detected a 10% decrease in the Mn^{2+} signal (Fig. 3, top spectrum). This result establishes that the dissociation constant (K_d) for the R-BIRK–Mn^{2+} complex is greater than 800 μM, consistent with the kinetic results in Fig. 1*B*.

A mixture of R-BIRK, Mg^{2+}, Mn^{2+}, and ATP was analyzed by EPR to determine whether conditions that permit autophosphorylation of R-BIRK will alter the interaction with Mn^{2+}. The results (Fig. 3, middle spectrum) indicate that under the conditions of the experiment, the free Mn^{2+} signal decreases to one-third that of the added Mn^{2+}. Control experiments without R-BIRK (Fig. 3, bottom spectrum) confirmed that the Mn^{2+} is bound to ATP rather than to R-BIRK. If R-BIRK had a high affinity for MnATP compared to MgATP, the MnATP complex that is formed would be sequestered on the enzyme, thereby depleting the solution of free Mn^{2+}. The results in Fig. 3 establish that phosphorylated R-BIRK does not bind tightly to free Mn^{2+} or MnATP.

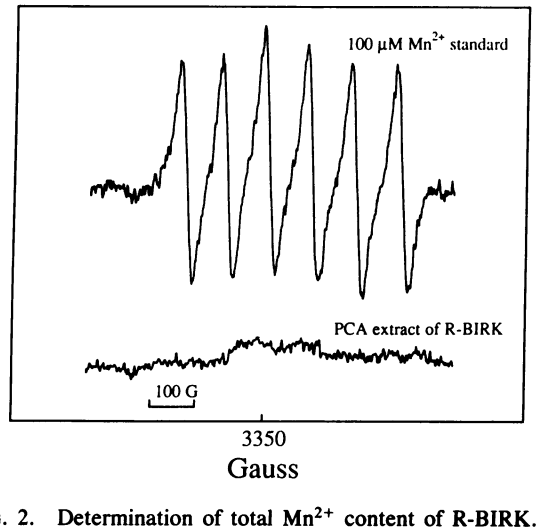

FIG. 2. Determination of total Mn^{2+} content of R-BIRK. The upper EPR spectral scan is from a standard of 100 μM $MnCl_2$ in 50 mM Tris/HCl (pH 7.4) containing 100 mM NaCl and 1 mM dithiothreitol. The lower EPR spectral scan represents the total Mn^{2+} in 95 μM R-BIRK. A sample of 100 μM R-BIRK, in the same buffer used for the upper spectrum, was denatured by the addition of 60% perchloric acid (PCA) to give a final concentration of 3% perchloric acid and 95 μM R-BIRK. Denatured protein was removed by centrifugation and the supernatant was analyzed for Mn^{2+} content by EPR. Receiver gain was equal for the upper and lower scans.

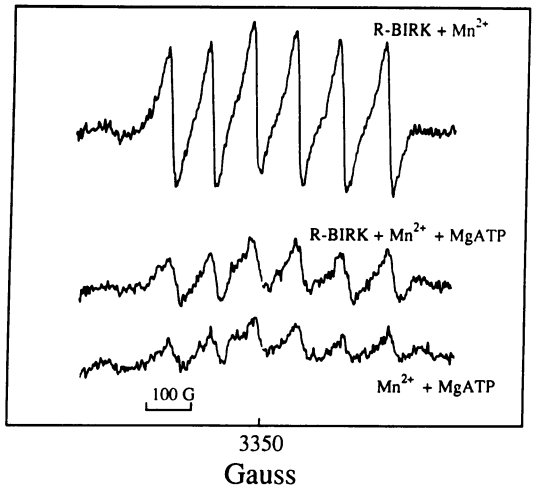

FIG. 3. Interaction of Mn^{2+} with R-BIRK. The top EPR spectral scan was obtained with a mixture of 88 μM R-BIRK and 59 μM $MnCl_2$ in 50 mM Tris/HCl (pH 7.4) containing 100 mM NaCl and 1 mM dithiothreitol. The middle scan was obtained with a mixture of 84 μM R-BIRK, 5.3 mM magnesium acetate, 1.1 mM ATP, and 56 μM $MnCl_2$. The bottom scan was obtained from a sample the same as for the middle scan, except that R-BIRK was replaced by the Tris buffer described above. The receiver gain was equal for all three scans.

Effect of Mn^{2+} on the CD Spectrum of R-BIRK. Villalba *et al.* (15) reported a preliminary spectroscopic characterization of the soluble kinase domain. From the results obtained with far-ultraviolet CD spectroscopy, they predicted the secondary structure content of R-BIRK to be 20.8% α-helix, 25.5% β-sheet, 9.9% β-turn, and 43.8% random coil. In these studies the presence of Mn^{2+} resulted in a change in the CD spectrum for R-BIRK. As shown in Fig. 4, the value for the mean residue-weight ellipticity at 207 nm increased with increasing concentrations of Mn^{2+} (between 0.5 and 4 mM). This correlates with a decrease in α-helical content from 20.8% to approximately 15%. The concentration of Mn^{2+} requisite to cause this effect agrees with the binding constant determined from EPR. Fig. 4 also shows that Mg^{2+} (up to 3 mM) did not result in any appreciable perturbation of the CD spectrum. When the intrinsic fluorescence emission spectra of R-BIRK (excitation at 295 nm) were monitored in the presence and absence of up to 5 mM Mn^{2+} or Mg^{2+}, there were no detectable spectral shifts (data not shown).

Fig. 5 displays the far-ultraviolet CD spectra of R-BIRK in

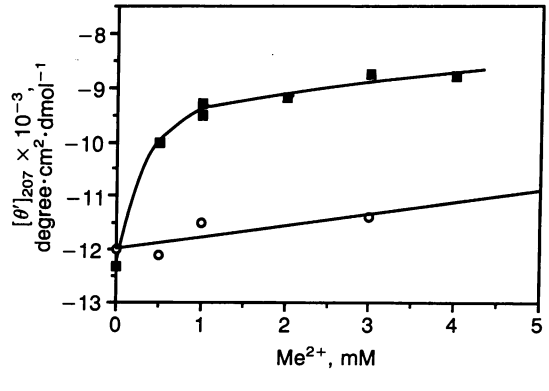

FIG. 4. Divalent cation concentration dependence of the mean residue-weight ellipticity of R-BIRK measured at 207 nm ($[\theta]_{207}$) by far-ultraviolet CD spectroscopy. The effect of divalent metal cation (Me^{2+}) concentration was measured with several independent preparations of R-BIRK (at protein concentration between 0.1 and 0.2 mg/ml) plus either Mn^{2+} (■) or Mg^{2+} (○). Additions of buffer to R-BIRK resulted in no change in the spectra.

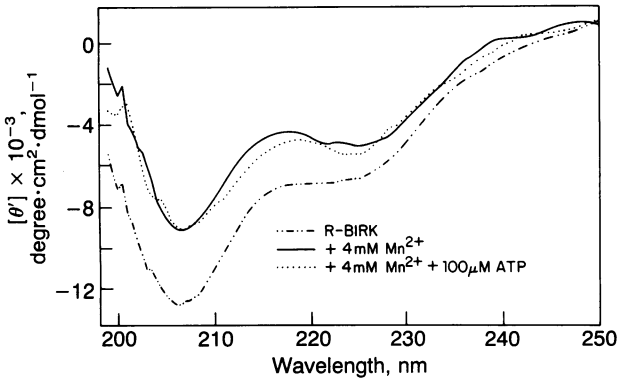

FIG. 5. The far-ultraviolet CD spectrum of R-BIRK. The spectra of R-BIRK (0.112 mg/ml) in 50 mM Tris/HCl buffer (pH 7.4) containing 100 mM NaCl were examined in the absence of ligands (– · · –), in the presence of 4 mM Mn^{2+} (——), and in the presence of 4 mM Mn^{2+} plus 100 μM ATP (·····). The spectra have been corrected for the buffer baselines and protein concentration.

the presence and absence of 4 mM Mn^{2+}. The dramatic change in the spectrum caused by Mn^{2+} binding is clear. However, the addition of ATP to the R-BIRK–Mn^{2+} complex did not result in any further perturbations of the spectrum. The data in Fig. 4 and Fig. 5 provide evidence that the binding of Mn^{2+} to R-BIRK, independent of Mg^{2+} and ATP, induces structural changes in the tyrosine kinase domain of the human insulin receptor.

DISCUSSION

R-BIRK retains many of the catalytic characteristics of the holoreceptor (15) and is therefore a model system for examining the structure and function of the cytoplasmic domain of the human insulin receptor. From the results presented in this report it is clear that Mn^{2+} is not tightly bound to R-BIRK following purification, and R-BIRK does not bind Mn^{2+} tightly *in vitro*. From direct EPR binding studies, and from the inferred dissociation constants of the kinetic activation and CD perturbations, Mn^{2+} has a K_d near 1 mM for R-BIRK. Because R-BIRK, and presumably the human insulin receptor, bind Mn^{2+} weakly, the intracellular concentration of free Mn^{2+} is clearly too low for Mn^{2+} binding to occur in intact cells. It is possible that in the cell, the insulin receptor tyrosine kinase interacts with the membrane and other factors in such a way that Mn^{2+} binding is much tighter. However the activation of native insulin receptor in membrane extracts appears to require similar Mn^{2+} concentrations (7–11).

These results are in agreement with the conclusions of previous enzymatic studies at high ATP concentrations (13, 14) and provide additional evidence that Mn^{2+} activation may not be a significant event in intact cells. We have also been able to directly test the published hypotheses for preferential binding of MnATP over MgATP, or an increased affinity for MnATP after Mn^{2+} binding (12–14). From the EPR studies when both the divalent cation–ATP complexes were present, the enzyme does not show a strong preference for MnATP binding over MgATP. The conformational change induced by Mn^{2+} binding to R-BIRK indicates that the mechanism of Mn^{2+} action may be physically different from that of Mg^{2+}.

The two *in vitro* consequences of the addition of Mn^{2+} to R-BIRK, tyrosine kinase activation and alteration of secondary structure, both occur under the same conditions with the same apparent K_d for Mn^{2+}. This Mn^{2+}-dependent kinase activation and coincident conformational change could reflect several kinds of phenomena *in vivo*. It seems unlikely that these Mn^{2+} effects are an artifact of purification, since crude extracts containing the human insulin receptor are also sensitive to activation by Mn^{2+} (7–11). It is possible that the *in vitro* conformation of R-BIRK in the presence of Mn^{2+} is actually the "activable" conformation of the tyrosine kinase domain in the intact cell membrane. This activable conformation may be that which is native to the membrane-bound cytoplasmic domain of the receptor. Present technology limits the ability to determine protein structure in the intact cell and provide documentation for this hypothesis.

It is also conceivable that Mn^{2+} mimics an actual effector that is present in intact cells. The existence of an activator has also been suggested by studies with poly(Lys), BIRK, and the human insulin receptor tyrosine kinases (18, 20). The addition of poly(Lys) allowed full activation in the presence of Mg^{2+} alone (0.4–4 mM) or Mn^{2+} at lower concentrations (0.2–0.4 mM). The effect of poly(Lys) on the CD spectrum of R-BIRK cannot be evaluated because this polymer possesses its own intrinsic CD spectrum. In preliminary fluorescence studies with R-BIRK, the presence of poly(Lys) (2–10 μg/ml; data not shown), as with Mn^{2+}, resulted in no perturbations in the intrinsic emission spectra of R-BIRK. A physiological activator [which Mn^{2+} and poly(Lys) may be mimicking] has yet to be identified.

In summary, studies of a purified recombinant insulin receptor protein-tyrosine kinase domain indicate that (*i*) kinase activity is stimulated by Mn^{2+} with a kinetic constant near 1 mM in the presence or absence of Mg^{2+}; (*ii*) the purified protein does not contain residual tightly bound Mn^{2+}; (*iii*) Mn^{2+} must bind to the protein weakly since it is not detected by EPR, which can measure dissociation constants of ≤500 μM; (*iv*) the far-ultraviolet CD spectrum of the protein is dramatically affected by the addition of Mn^{2+}, with an apparent K_d near 1 mM, but the intrinsic fluorescence pattern is not; and (*v*) the concentrations of Mn^{2+} required to see effects *in vitro* are supraphysiological, suggesting that free Mn^{2+} is unlikely to be an activator in intact cells.

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