Differential sensitivity of the insulin-receptor kinase to thiol and oxidizing agents in the absence and presence of insulin

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The purified human placental insulin-receptor β -subunit autophosphorylating activity was found to be inhibited, in a time- and concentration-dependent manner, by the specific thiol-alkylating agents *N*-ethylmaleimide and 5,5'-dithiobis-(2-nitrobenzoic acid). The insulin-receptor kinase was observed to be more sensitive to inhibition by *N*-ethylmaleimide in the presence [IC_{50} (concn. giving 50% inhibition) = $25 \pm 3 \mu M$] than in the absence ($IC_{50} = 73 \pm 6 \mu M$) of insulin. Similarly, inhibition by 5,5'-dithiobis-(2-nitrobenzoic acid) occurred with $IC_{50} = 30 \pm 6 \mu M$ in the presence and $155 \pm 35 \mu M$ in the absence of insulin. Examination of the exogenous-substrate protein kinase activity demonstrated that the differential sensitivity to *N*-ethylmaleimide was due to direct inhibition of protein kinase activity, as opposed to blockade of the phospho-acceptor properties of the insulin receptor. In contrast, iodoacetamide had essentially no effect on the insulin-receptor β -subunit autophosphorylating activity and was able to protect partially against the *N*-ethylmaleimide inhibition in both the presence and the absence of insulin. Consistent with these findings, none of the thiol-specific agents were able to alter significantly insulin binding at concentrations which maximally inhibited the β -subunit autophosphorylation. Further, in the presence of insulin, the insulin-receptor kinase activity was also observed to be more sensitive to oxidation by H_2O_2 and $FeCl_3$ /ascorbate compared with insulin receptors in the absence of insulin. These results indicate that there is a critical thiol group(s) necessary for the β -subunit autophosphorylating activity of the insulin-receptor kinase and that in the presence of insulin is more susceptible to exogenously added thiol and oxidizing agents.

INTRODUCTION

The insulin receptor has been well established to exist minimally as a heterotetrameric disulphide-linked complex consisting of two M_r -130 000 (α) and two M_r -95 000 (β) subunits (Hedo *et al.*, 1981; Massague *et al.*, 1981; Van Obberghen *et al.*, 1981; Fujita-Yamaguchi, 1984; Boyle *et al.*, 1985). It is thought that the α subunit encompasses the high-affinity insulin-binding site (Yip *et al.*, 1978, 1980; Jacobs *et al.*, 1979; Pilch & Czech, 1980; Yeung *et al.*, 1980), whereas the β subunit contains a single hydrophobic transmembrane domain (Ebina *et al.*, 1985; Ullrich *et al.*, 1985), an intracellular ATP-binding site (Roth & Cassell, 1983; Shia & Pilch, 1983; Van Obberghen *et al.*, 1983), as well as tyrosine-autophosphorylation receptor sites (Avruch *et al.*, 1982; Kasuga *et al.*, 1982a,b,c, 1983a,b; Petruzzelli *et al.*, 1982, 1984; Shia & Pilch, 1983; Tamura *et al.*, 1983; Zick *et al.*, 1983).

The insulin-receptor kinase has been observed to be activated by reducing agents, e.g. dithiothreitol (DTT) (Shia *et al.*, 1983; Fujita-Yamaguchi & Kathuria, 1985; Sweet *et al.*, 1986), and to be inhibited by the specific thiol-alkylating agent *N*-ethylmaleimide (NEM) (Shia *et al.*, 1983; Zick *et al.*, 1983; Pike *et al.*, 1984; Wilden *et al.*, 1986). We have also observed that the DTT-dependent activation of the insulin-receptor kinase is additive with that of insulin (Wilden *et al.*, 1986). Further, insulin was found to potentiate the DTT-dependent reduction of the insulin-receptor $\alpha_2\beta_2$ disulphide-linked complex, when analysed by SDS/polyacrylamide-gel electrophoresis. These observ-

ations strongly imply that intramolecular oxidation-reduction reactions may be a possible regulatory mechanism for the intramolecular transmembrane-signal activation of the insulin-receptor kinase. In this paper, we report the effects of several thiol and oxidizing agents on the insulin-receptor β -subunit autophosphorylating activity. These results suggest that insulin induces an alteration of the insulin-receptor structure which results in a greater susceptibility of a critical thiol group(s) to thiol and oxidizing agents.

EXPERIMENTAL

Materials

n-Octyl β -D-glucopyranoside, proteinase inhibitors, thiol reagents, M_r standards, ATP and Triton X-100 were purchased from Sigma. Affi-Gel 10 and reagent for SDS/polyacrylamide-gel electrophoresis were purchased from Bio-Rad. XAR-5 film and Cronex lightning and intensifying screens were obtained from Eastman Kodak. Sephacryl S-400, Centricon 30 micro-concentrators and NCS tissue solubilizer were purchased from Pharmacia, Amicon and Amersham respectively. [γ - ^{32}P]ATP (3000 Ci/mmol) was purchased from New England Nuclear. Pig insulin was a gift from Dr. R. Chance, Eli Lilly Co. Monoiodinated (residue A¹⁴) pig insulin was kindly provided by the Diabetes Endocrinology Research Center, University of Iowa.

Purification of insulin receptor

Insulin receptors were purified from freshly obtained

full-term human placenta as previously described (Boyle *et al.*, 1985; Sweet *et al.*, 1985). Briefly, placental membranes were prepared as described by Harrison & Itin (1980). The membranes were solubilized in 2.0% Triton X-100 for 1 h at 4 °C in 10 mM-Tris/HCl (pH 8.0)/250 mM-sucrose/2 mM-EDTA and proteinase inhibitors. Insulin receptors were then purified by sequential chromatography on Sephacryl S-400 and insulin-agarose. The eluate from the insulin-agarose was concentrated on Centricon 30 micro-concentrators, washed with 50 mM-Hepes (pH 8.0)/10% (v/v) glycerol/0.6% n-octyl β -D-glucopyranoside, and stored at 4 °C until used.

Insulin-receptor autophosphorylation

Insulin receptors were incubated for 1 h in the absence or presence of 200 nM-insulin in 50 mM-Hepes (pH 8.0)/10 mM-MgCl₂/10 mM-MnCl₂. The phosphorylation reaction was initiated by the addition of [γ -³²P]ATP (1.0 mM-ATP, 3.0 μ Ci/nmol) to give a final concentration of 100 μ M-ATP. The reaction was allowed to continue for 5 min at 23 °C and was terminated by the addition of Laemmli (1970) sample buffer (50 mM-Tris/HCl, pH 6.9, 10% glycerol, 0.05% Bromophenol Blue and 1.0% SDS) containing 100 mM-DTT and subjected to SDS/polyacrylamide-gel electrophoresis.

SDS/polyacrylamide-gel electrophoresis

One-dimensional gel electrophoresis was performed in a 7%-acrylamide separating gel and a 3% stacking gel (acrylamide/bisacrylamide, 37.5:1) as described by Laemmli (1970). The gels were fixed, stained, destained, dried and autoradiographed on Kodak XAR-5 film with Cronex lightning plus intensifying screens. The M_r -95000 β -subunit band was excised, rehydrated, solubilized with NCS tissue solubilizer, mixed with scintillation solution and counted for ³²P.

Insulin binding

Insulin receptors were incubated with 0.25 nM-¹²⁵I-

insulin in a final volume of 0.2 ml of Krebs-Ringer Hepes buffer (50 mM-Hepes, 110 mM-NaCl, 5.1 mM-KCl, 1.3 mM-CaCl₂, 1.3 mM-MgSO₄), pH 7.4, plus 0.1% bovine serum albumin for 45 min at 23 °C. Free ¹²⁵I-insulin was separated from bound hormone by the standard poly(ethylene glycol)-precipitation method (Cuatrecasas, 1972). Non-specific binding, determined in the presence of 15 μ M unlabelled insulin, ranged from 3 to 5% of the total binding.

Exogenous-substrate phosphorylation

Insulin receptors were treated as described in the Figure legends, and the kinase reactions were initiated by the addition of 0.1 mg of poly(Glu:Tyr) (4:1) and [γ -³²P]ATP (1.0 mM-ATP, 3 μ Ci/nmol), as previously described. The reactions were terminated by the addition of samples on to Whatman 3MM filter paper and immersion in ice-cold 10% (v/v) trichloroacetic acid/10 mM-Na₂HPO₄. Filters were extensively washed in the above buffer, dried, scintillation fluid was added, and they were counted for ³²P.

RESULTS

We and others have previously reported that the specific thiol-alkylating agent NEM can inhibit the β -subunit autophosphorylation of the insulin receptor in a time- and concentration-dependent manner (Shia *et al.*, 1983; Zick *et al.*, 1983; Pike *et al.*, 1984; Wilden *et al.*, 1986). Further, the insulin-receptor kinase activity was observed to be less sensitive to NEM inhibition in the absence of insulin than in the presence of insulin (Shia *et al.*, 1983; Wilden *et al.*, 1986). Consistent with these previous studies, the insulin-stimulated β -subunit autophosphorylation of the purified human placental insulin receptor was found to be more susceptible to NEM inhibition than the untreated insulin receptors in both a time- and concentration-dependent manner (Fig. 1). The half-maximal NEM inhibition of the initial rate of β -subunit autophosphorylation in the presence of insulin

Fig. 1. Time- and concentration-dependent inhibition by NEM of [γ -³²P]ATP β -subunit autophosphorylation of the insulin-receptor kinase

Insulin receptors (0.5 μ g) were incubated in the absence (●) or presence (○) of 200 nM-insulin for 1 h at 23 °C. Samples were then incubated with various concentrations of NEM for 1 h at 23 °C (a) or with 100 μ M-NEM for various times at 23 °C (b). Autophosphorylation was carried out for 5 min at 23 °C as described in the Experimental section. It has previously been demonstrated that under these conditions the reaction rates are linear, thus ensuring measurements of the initial rates of β -subunit autophosphorylation (Zick *et al.*, 1983; Nemenoff *et al.*, 1984). Laemmli (1970) sample buffer containing 100 mM-DTT was added before electrophoresis in a SDS/7% -polyacrylamide gel. The gels were processed for autoradiography. The β -subunit was excised, hydrated, solubilized and prepared for scintillation counting as described in the Experimental section.

Fig. 2. Time- and concentration-dependent inhibition by DTNB of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ β -subunit autophosphorylation of the insulin-receptor kinase

Insulin receptors ($0.5\text{ }\mu\text{g}$) were incubated in the absence (●) or presence (○) of 200 nM -insulin for 1 h at $23\text{ }^{\circ}\text{C}$. Samples were then incubated with various concentrations of DTNB for 1 h at $23\text{ }^{\circ}\text{C}$ (a) or with 1 mM -DTNB for various lengths of time at $23\text{ }^{\circ}\text{C}$ (b). Autophosphorylation was carried out for 5 min at $23\text{ }^{\circ}\text{C}$ as described in the Experimental section. Laemmli (1970) sample buffer containing 100 mM -DTT was added before electrophoresis in a SDS/7%-polyacrylamide gel. The gels were processed for autoradiography. The β -subunit was excised, hydrated, solubilized and prepared for scintillation counting as described in the Experimental section.

Fig. 3. Time- and concentration-dependent inhibition by NEM of insulin-receptor kinase activity toward exogenous substrate

Insulin receptors ($0.5\text{ }\mu\text{g}$) were incubated in the absence (●) or presence (○) of 200 nM -insulin for 1 h at $23\text{ }^{\circ}\text{C}$. Samples were then incubated for 1 h at $23\text{ }^{\circ}\text{C}$ with various concentrations of NEM before the kinase reaction (a). Alternatively, samples were then incubated with $100\text{ }\mu\text{M}$ -NEM at $23\text{ }^{\circ}\text{C}$ for various times before the kinase reaction (b). Phosphorylation was carried out for 15 min at $23\text{ }^{\circ}\text{C}$ as described in the Experimental section. It has previously been demonstrated that under these conditions the reaction rates are linear, thus ensuring measurements of the initial rate of exogenous-substrate phosphorylation (Rosen *et al.*, 1983).

occurred at $25 \pm 3\text{ }\mu\text{M}$, whereas in the absence of insulin it occurred at $73 \pm 6\text{ }\mu\text{M}$ (Fig. 1a). The half-time for NEM inhibition of the β -subunit autophosphorylation ($100\text{ }\mu\text{M}$ -NEM) in the presence of insulin was approx. 15 min , whereas in the absence of insulin it was approx. 30 min (Fig. 1b).

Similarly, the thiol-specific reagent DTNB was also observed to inhibit the insulin-receptor β -subunit autophosphorylation in a concentration- (Fig. 2a) and time- (Fig. 2b) dependent manner. The half-maximal DTNB inhibition of β -subunit autophosphorylation in the presence of insulin was at $30 \pm 6\text{ }\mu\text{M}$, whereas in the absence of insulin it occurred at $155 \pm 35\text{ }\mu\text{M}$. The half-time for DTNB (1 mM) inhibition of the β -subunit autophosphorylation in the presence of insulin was also approx. 15 min , whereas in the absence of insulin it was approx. 30 min .

To determine if the differential sensitivity of the insulin-receptor kinase to thiol agents was due to inhibition of enzymic activity or to inhibition of the insulin receptor as a phospho-acceptor, we next examined the insulin-receptor exogenous-substrate protein kinase activity (Fig. 3). NEM was again observed to inhibit in a concentration- (Fig. 3a) and time- (Fig. 3b) dependent manner, the initial rate of substrate [poly(Glu:Tyr)] phosphorylation. Similarly to the β -subunit autophosphorylation, the half-maximal NEM inhibition of poly(Glu:Tyr) phosphorylation in the presence of insulin occurred at $20\text{ }\mu\text{M}$, whereas in the absence of insulin it was at $100\text{ }\mu\text{M}$. The half-time for NEM ($100\text{ }\mu\text{M}$) inhibition of the poly(Glu:Tyr) phosphorylation in the presence of insulin was approx. 45 min , whereas in the absence of insulin it was 70 min .

In contrast with the inhibition observed by NEM and

Fig. 4. Concentration-dependent inhibition by iodoacetamide of [γ - 32 P]ATP β -subunit autophosphorylation of the insulin-receptor kinase

Insulin receptors ($0.5 \mu\text{g}$) were incubated in the absence (●) or presence (○) of 200 nM-insulin for 1 h at 23 °C. Samples were incubated with various concentrations of iodoacetamide for 1 h at 23 °C. Autophosphorylation was carried out for 5 min at 23 °C as described in the Experimental section. Laemmli (1970) sample buffer containing 100 mM-DTT was added before electrophoresis in a SDS/7% -polyacrylamide gel. The gels were processed for autoradiography. The β -subunit was excised, hydrated, solubilized and prepared for scintillation counting as described in the Experimental section.

DTNB, the specific thiol reagent iodoacetamide was unable to inhibit the insulin-receptor β -subunit autophosphorylation in either the presence or the absence of insulin (Fig. 4). At the highest concentration of iodoacetamide examined (10 mM), essentially no effect on the insulin-receptor β -subunit autophosphorylation was detectable.

To determine if the iodoacetamide was reacting with the insulin receptor, we examined the ability of iodoacetamide to protect the β -subunit autophosphorylation activity from NEM inhibition (Fig. 5). In the absence of insulin, 100 μM -NEM inhibited the β -subunit autophosphorylation by 60% (Fig. 5a). Treatment of the insulin receptor with 100 μM -NEM, followed by 1.0 mM-iodoacetamide, also resulted in a similar inhibition (63%) of β -subunit autophosphorylation. As previously observed (Fig. 4), treatment of the insulin receptor with 1.0 mM-iodoacetamide had essentially no effect on the β -subunit autophosphorylating activity. However, treatment of the insulin receptor with 1.0 mM-iodoacetamide before incubation with 100 μM -NEM resulted in a marked decrease (79%) in the ability of NEM to inhibit the β -subunit autophosphorylation.

Similarly to the studies in the absence of insulin, insulin receptors which were treated with insulin before NEM addition showed a 50% inhibition of the β -subunit autophosphorylation. Further, iodoacetamide treatment had essentially no effect on the β -subunit autophosphorylation (Fig. 5b). In contrast, incubation with 1.0 mM-iodoacetamide after insulin treatment markedly

Fig. 5. Iodoacetamide protection against NEM inhibition of the [γ - 32 P]ATP β -subunit autophosphorylation of the insulin receptor

Insulin receptors ($0.5 \mu\text{g}$) were incubated with 100 μM -NEM, 1 mM-iodoacetamide (IAN) and 200 nM-insulin (INS), for 30 min each, in the order shown in the Figure. Autophosphorylation was carried out for 5 min at 23 °C as described in the Experimental section. Laemmli (1970) sample buffer containing 100 mM-DTT was added to the samples before electrophoresis on a SDS/7% -polyacrylamide gel. The gels were then processed for autoradiography. The β -subunit was prepared for scintillation counting as described in the Experimental section; Panels (a) and (b) show experiments without and with insulin respectively, and 100% represents the ^{32}P c.p.m. incorporated into the β subunit of the insulin receptor without or with initial treatment with 200 nM-insulin. The stimulation by insulin over the non-insulin-treated insulin-receptor kinase ranged from 2–3.5-fold in four trials. The error bars represent S.E.M. values, for $n = 3$ in (a) and $n = 4$ in (b).

decreased the NEM inhibition of β -subunit autophosphorylation (76%). Further, iodoacetamide pretreatment did not alter the ability of insulin to stimulate the insulin-receptor β -subunit autophosphorylating activity, even though it was still as effective in protecting against the NEM inhibition. Under the conditions of

Fig. 6. Effect of thiol reagents on ^{125}I -insulin binding to the insulin receptor

Insulin (a) or the insulin receptor (b) was preincubated with various concentrations of thiol reagents NEM (○), DTNB (□) or iodoacetamide (△) for 45 min at 23 °C. Specific insulin binding was determined as described in the Experimental section. Control binding was determined without the preincubation with the thiol reagent.

these experiments, insulin was found to increase the β -subunit autophosphorylation approx. 2.5-fold.

We have previously reported that, although the insulin-receptor α subunit has 3 times the number of cysteine residues compared with the β subunit (Ebina *et al.*, 1985; Ullrich *et al.*, 1985), only the β subunit is susceptible to [^3H]NEM labelling at low concentrations (Wilden *et al.*, 1986). Consistent with these results, treatment of the insulin receptors with NEM, DTNB or iodoacetamide, up to 1.0 mM, had no significant effect on insulin binding (Fig. 6a). At higher concentrations of these thiol reagents (10 mM) a small decrease in insulin binding was observed. In similar experiments, pretreatment of the ^{125}I -insulin with NEM, DTNB or iodoacetamide before incubation with the insulin receptor also resulted in no significant change in insulin-binding activity at concentrations of these thiol reagents below 1 mM (Fig. 6b).

We next investigated the effect of several oxidizing agents on the insulin-receptor β -subunit autophosphoryl-

Fig. 7. H_2O_2 inhibition of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ β -subunit autophosphorylation of the insulin receptor

Insulin receptors (1.0 μg) were incubated in the presence (○) or absence (●) of 200 nM-insulin for 1 h at 23 °C. The samples were then treated with 100 μM - H_2O_2 at 23 °C for the times shown in the Figure. Autophosphorylation was carried out for 5 min at 23 °C as described in the Experimental section. Laemmli (1970) sample buffer containing 100 mM-DTT was added to each sample before electrophoresis in a SDS/7% -polyacrylamide gel. The gels were processed for autoradiography. The β subunit was prepared for scintillation counting as described in the Experimental section. Percentage inhibition of the insulin-receptor β -subunit autophosphorylation as a function of time in the presence of 100 μM - H_2O_2 is shown in the inset.

ating activity. A time-dependence of insulin receptors in the presence of H_2O_2 is shown in Fig. 7. Oxidation of the insulin receptor by H_2O_2 appeared to indicate an increased sensitivity of β -subunit autophosphorylation in the presence of insulin compared with that in the absence of insulin (Fig. 7, inset). However, owing to the speed with which H_2O_2 inhibits the insulin-receptor β -subunit autophosphorylation it was difficult clearly to observe a time-dependence. We therefore used the mixed-function-oxidation system of FeCl_3 /ascorbate, which allows finer control of the rate of oxidation of the insulin receptor. A time-dependence of insulin receptors in the presence of this mixed-function-oxidation system is shown in Fig. 8. FeCl_3 /ascorbate oxidation of the insulin receptor clearly demonstrated an increased sensitivity of β -subunit autophosphorylation in the presence of insulin compared with that in the absence of insulin. The half-time for the inhibition of β -subunit autophosphorylation under these conditions was approx. 20 and 40 min in the presence and the absence of insulin respectively.

DISCUSSION

It has been previously established that the specific thiol reagent NEM can inhibit the tyrosine-specific β -subunit autophosphorylation and exogenous-protein substrate

Fig. 8. FeCl_3 /ascorbate inhibition of the $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ β -subunit autophosphorylation of the insulin receptor

Insulin receptors ($1.0\ \mu\text{g}$) were incubated in the presence (○) or absence (●) of $200\ \text{nM}$ -insulin for 1 h at 23°C . Subsequently the insulin receptors were incubated with $0.1\ \text{mM}$ - FeCl_3 / $2.0\ \text{mM}$ -L-ascorbate for the times shown at 23°C , and the reactions were terminated by addition of $3\ \mu\text{g}$ of catalase. Autophosphorylation was carried out for 5 min at 23°C as described in the Experimental section. Laemmli (1970) sample buffer containing $100\ \text{mM}$ -DTT was added to each sample before electrophoresis in a SDS/7%-polyacrylamide gel. The gels were processed for autoradiography. The β subunit was prepared for scintillation counting as described in the Experimental section. Percentage inhibition of the insulin-receptor β -subunit autophosphorylation as a function of time in the presence of FeCl_3 /ascorbate is shown in the inset.

phosphorylation of the insulin-receptor kinase (Shia *et al.*, 1983; Zick *et al.*, 1983; Pike *et al.*, 1984). Further, the insulin-receptor protein kinase activity was observed to be more sensitive to NEM inhibition in the presence of insulin than in its absence (Shia *et al.*, 1983; Wilden *et al.*, 1986). These previous studies have led us to examine the effects of other thiol and oxidizing agents on the insulin-receptor β -subunit autophosphorylating activity. The data in Fig. 1 document the increased NEM-sensitivity of β -subunit autophosphorylation in the presence of insulin compared with in its absence, as previously reported. Similarly, DTNB also was found to inhibit the insulin-stimulated β -subunit autophosphorylation at an approx. 5-fold lower concentration than in the absence of insulin (Fig. 1). We have also demonstrated an appropriate time-dependence for the inhibition of insulin-receptor β -subunit autophosphorylation in the presence and absence of insulin at a fixed concentration of thiol reagent.

The differential sensitivity of the insulin-receptor kinase activity toward exogenously added substrate is shown by the similar concentration- and time-dependence effect of NEM. These studies argue against the possibility that the thiol reagents were affecting the

phospho-acceptor portion of the insulin receptor, since only effects of the thiol reagents on the enzymic kinase activity of the insulin receptor were determined in the experiments involving exogenously added substrate.

In contrast, similar studies performed with the thiol-specific reagent iodoacetamide demonstrated no alteration in the β -subunit autophosphorylation in either the presence or the absence of insulin. In order to ascertain whether or not iodoacetamide was actually reacting with the insulin receptor at the same site(s) as NEM, we examined the ability of iodoacetamide to protect against the NEM inhibition of β -subunit autophosphorylation. The data in Fig. 5 demonstrate that iodoacetamide can partially protect against the NEM inhibition of β -subunit autophosphorylation in both the presence and the absence of insulin. These results suggest that the same critical thiol group(s) which are susceptible to NEM alkylation are also available to react with iodoacetamide. The lack of insulin-receptor kinase inhibition by iodoacetamide may be accounted for by the fact that this thiol reagent is a much smaller molecule than either NEM or DTNB, and therefore may not significantly alter the insulin-receptor structure. Alternatively, after the thiol reaction with iodoacetamide the critical thiol group(s) may still be able to participate in appropriate hydrogen bonding necessary for the kinase activity. This latter alternative appears unlikely, since iodoacetic acid treatment of the insulin receptor was also observed to have no significant effect on the insulin-receptor kinase activity (P. A. Wilden & J. E. Pessin, unpublished work).

We have previously reported that only the β subunit of the insulin receptor kinase is susceptible to $[\text{H}]\text{NEM}$ (Wilden *et al.*, 1986) labelling, although the α subunit contains 3 times the number of cysteine residues in the primary amino acid sequence (Ebina *et al.*, 1985; Ullrich *et al.*, 1985). Consistent with this previous observation, preincubation of the insulin receptor with the various thiol reagents (NEM, DTNB or iodoacetamide) did not alter insulin binding over the concentration range which inhibits the β -subunit autophosphorylating activity (Figs. 6a and 6b). However, at much higher concentrations of these thiol reagents, a small decrease of insulin-binding activity was observed. This decrease cannot account for the inhibition of β -subunit autophosphorylating activity.

Related studies have shown that the β -subunit autophosphorylation of the insulin receptor is stimulated by reducing agents such as DTT and thioredoxin (Shia *et al.*, 1983; Fujita-Yamaguchi & Kathuria, 1985; Sweet *et al.*, 1986; Wilden *et al.*, 1986). The observations that reduction activates and that specific thiol reagents inhibit the insulin-receptor kinase activity implied to us that oxidizing agents should also inhibit the insulin-receptor β -subunit autophosphorylation. This appears to be the case for H_2O_2 and is more clearly shown in the mixed-oxidation system of FeCl_3 /ascorbate. Oxidation inhibits the insulin-stimulated β -subunit autophosphorylation at a greater rate than the untreated insulin-receptor preparations (Fig. 8). These data further support the hypothesis that, in the presence of insulin, an essential thiol group(s) has become more accessible to exogenously added reagents. Further elucidation of the critical structural elements of the insulin receptor are necessary future objectives in order to determine the molecular events responsible for the insulin-dependent trans-

membrane activation of the β -subunit protein kinase domain.

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