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Binding of a Glucagon Photoaffinity Label to Rat Liver Plasma Membranes and Its Effect on Adenylate Cyclase Activity before and after Photolysis†

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ABSTRACT: The concentration-dependent stimulation of adenylate cyclase by the photoaffinity reagent 2-[(2-nitro-4-azidophenyl)sulfenyl]-Trp^{25}-glucagon (glucagon-NAPS) and also its binding characteristics were compared with those of the native hormone. The derivative was found to be slightly more potent in stimulating adenylate cyclase than glucagon, in the presence of guanosine 5′-triphosphate (GTP). ^{125}I-Labeled glucagon-NAPS or ^{125}I-labeled glucagon bound rapidly to receptors and was competitively displaced by unlabeled glucagon or glucagon-NAPS. Glucagon-NAPS displaced bound radiolabeled hormone at a lower concentration than did glucagon in the absence of GTP. Scatchard analysis of the binding data obtained from displacement of bound radiolabeled ligand with unlabeled peptide demonstrated a heterogeneous population of saturable glucagon binding sites. Glucagon-NAPS displayed a higher affinity (0.7 nM) for the high-capacity sites (80–90% of total binding sites) than glucagon (4.0 nM) in the absence of GTP. In the presence of the nucleotide, both ligands had approximately the same affinity (0.5–0.6 nM). Hill plot analysis of the binding data suggested noncooperative interactions. Photoaffinity labeling of plasma membranes with glucagon-NAPS resulted in an irreversible activation of adenylate cyclase with a reduced response to further stimulation by glucagon, glucagon-NAPS, and NaF.

One of the important mechanisms by which peptide hormones can initiate cellular response is through the binding of the hormone to specific, saturable receptor sites on the surface of plasma membranes of target cells (Insel, 1978; Levitzki & Helmreich, 1979; Rodbell, 1980). In the case of glucagon, it has been shown that the hormone binds reversibly to specific sites in liver plasma membranes at concentrations comparable to those required for stimulation of adenylate cyclase (Rodbell et al., 1971a, 1974; Lad et al., 1977; Welton et al., 1977). Guanosine 5′-triphosphate (GTP)[1] also affects adenylate cyclase activity and is required for activation by glucagon (Rodbell et al., 1971c, 1974). GTP lowers the affinity of the receptor for the hormone (Birnbaumer & Pohl, 1973; Lin et al., 1977).

Earlier studies from our laboratory have indicated that the hydrophobic amino acids of glucagon can be modified without loss of activity provided that the product is not polar (Epand, 1980; Epand et al., 1981). Nitrophenylsulfenyl derivatives of glucagon modified at the tryptophan-25 residue stimulated adenylate cyclase with the same or even higher potency than native glucagon (Epand & Cote, 1976; Wright & Rodbell, 1980). This property led to the synthesis of the photoreactive glucagon-NAPS derivative having a nitrophenyl azide moiety bound to position 2 of the indole ring of tryptophan at amino acid residue 25 of glucagon (Demoliou & Epand, 1980). Our purpose was to use this derivative for the photoaffinity labeling of the glucagon receptor. The criteria and advantages in using photoaffinity labeling over conventional affinity labeling have been discussed by Knowles (1972) and Bayley & Knowles (1977). Chemical modification of ligands with photoaffinity reagents has provided radiolabeled photoprobes which when

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[1] Abbreviations: NAPSCl, (2-nitro-4-azidophenyl)sulfenyl chloride; glucagon-NPS, 2-[(2-nitrophenyl)sulfenyl]-Trp^{25}-glucagon; NAP-glucagon, N^{α}-(4-azido-2-nitrophenyl)glucagon; glucagon-NAPS, 2-[(2-nitro-4-azidophenyl)sulfenyl]-Trp^{25}-glucagon; ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-phosphate; GTP, guanosine 5′-triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; BSA, bovine serum albumin; KIU, kallikrein inactivator units; NaDodSO$_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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irradiated give covalently linked ligand–receptor complexes that can be isolated and characterized more easily than purely reversible complexes (Bayley & Knowles, 1977; Das & Fox, 1979; Chowdhry & Westheimer, 1979; Hanstein, 1979). An important advantage in photoaffinity labeling is that the ligand–receptor interactions can be characterized prior to covalent labeling. The efficacy of nitrenes in photolabeling lipids and hydrophobic amino acid side chains of membrane proteins has been questioned recently (Bayley & Knowles, 1978a,b). However, successful photolabeling of hormone receptor molecules with phenyl azide photoprobes has been reported. Some examples are the photoaffinity labeling of putative receptors of enkephalin (Lee et al., 1979; Hazum et al., 1979), oxytocin (Stadel et al., 1978), corticotropin (Ramachandran et al., 1980), and insulin (Yip et al., 1978, 1980; Jacobs et al., 1979; Wisher et al., 1979; Yeung et al., 1980; Kuehn et al., 1980). Photoaffinity labeling of the glucagon receptor has been attempted by Bregman & Levy (1977). However, although the photoprobe could specifically displace membrane-bound radiolabeled glucagon, it was inactive in stimulating adenylate cyclase. Photoaffinity labeling of the glucagon receptor identified a membrane component of 25 000–27 000 daltons. Recently, Johnson et al. (1981) have identified a 53 000 molecular weight receptor component by using a photoactivated cross-linking reagent to attach ^{125}I-labeled glucagon to rat liver membranes.

In the effort to define and covalently label the glucagon receptor components in rat liver plasma membranes, in situ, it was considered important to demonstrate that adenylate cyclase stimulations by glucagon-NAPS and binding to plasma membranes in the absence of light were comparable to those of the native glucagon. In the studies concerning the physical properties of the derivative, we have demonstrated that ^{125}I-labeled glucagon-NAPS has the necessary chemical properties for photolabeling by nonsepcific covalent labeling of bovine serum albumin and specific covalent labeling of a glucagon-specific antibody (Demoliou & Epand, 1980). In the present studies, we demonstrate that the derivative has the required biological properties to bind to and to react with the specific receptor site necessary for the glucagon stimulation of cyclic AMP production. Characterization of the covalently labeled sites by $NaDodSO_4$–polyacrylamide gel electrophoresis is presented in the following paper (Demoliou-Mason & Epand, 1982).

Experimental Procedures

Materials

The synthesis and purification of (2-nitro-4-azidophenyl)-sulfenyl chloride (NAPSCl) and of glucagon-NAPS have been previously reported (Demoliou & Epand, 1980). Glucagon and glucagon-NAPS were iodinated by the lactoperoxidase method, and the monoiodo derivative was purified as previously described (Demoliou & Epand, 1980). Bovine serum albumin (fraction V), 98–100% grade creatine phosphate, rabbit muscle type I creatine phosphokinase, adenosine 5′-triphosphate, guanosine 5′-triphosphate, bacitracin, epinephrine, isoproterenol, iodoacetamide, iodoacetic acid, and aminophylline were from Sigma; [8-^{14}C]ATP (40–60 mCi/mmol), [2,8-^{3}H]cAMP (30–50 Ci/mmol), and Na^{125}I (17 Ci/mg) were from New England Nuclear; lactoperoxidase (260 units/mg, OD_{412}/OD_{280} = 0.74) and Trasylol (10 000 KIU/mL) were from Boehringer; potassium phthalate was from Matheson Coleman and Bell; bovine–porcine glucagon was purchased from Elanco Corp. The purity of the product was evaluated by partition chromatography as previously reported (Demoliou & Epand, 1980). All other reagents used were of the highest purity available commercially.

Methods

Membrane Purification. Partialy purified rat liver plasma membranes were prepared by minor modification of the Neville procedure (Demoliou & Epand, 1980) and were stored in a pelleted form at −70 °C. Their purity was evaluated by the use of marker enzymes.

Adenylate Cyclase Assay. Adenylate cyclase was assayed by the technique of Krishna et al. (1968) with [8-^{14}C]ATP as substrate and [2,8-^{3}H]cAMP as a tracer. The final assay medium contained 30 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 1 mM EGTA, 2 mM mercaptoethanol, 0.2 mM cAMP, 0.1 mM GTP, 0.56 mM [8-^{14}C]ATP, 5 mM creatine phosphate, 0.64 mg/mL creatine phosphokinase (50–60 units/mL), 1 mM aminophylline, 7 mM bacitracin, and 4 mg of BSA/mL in a final volume of 100 μL. Glucagon, glucagon-NAPS, or NaF was added as a solution in 30 mM Tris-HCl, pH 7.0, containing 1 mg of BSA/mL. When the activities of epinephrine and isoproterenol were tested, these activating agents, as well as glucaon, glucagon-NAPS, and NaF, were prepared in the same buffer containing 0.1% (w/v) ascorbate. The assay was carried out as previously reported (Demoliou & Epand, 1980), isolating [8-^{14}C]cAMP by the procdure of White & Karr (1978). Adenylate cyclase assays in the presence of glucagon-NAPS were performed in the dark under red light.

Irradiation Studies. Irradiation studies were carried out in a jacketed quartz cell (1.0-cm path length, 1.5-mL capacity) contained within a cast iron, black cell holder, held at 27 cm from a 7 mm arc length, 1000-W xenon/mercury lamp placed in an LH 15 N/N lamp housing and connected to a power supply (250–1000 W, Shoeffer Instrument Corp. Model LPS 255 HR). A 5-cm path-length 0.1% (w/v) potassium phthalate filter contained in a Pyrex cell (5 × 5 cm) and two ultraviolet-transmitting, visible-absorbing color filters (7–51 and 4.9–5.1 mm, Glass No. 4970, Corning, 80% transmittance at λ_{max} 365 nm) were used between the light source and the cell. Samples precooled in ice in the dark were purged with water (4 °C)-saturated N_2 gas for 1–2 min before irradiation. N_2 gas was blown through the sample during photolysis at a sufficient rate to ensure mixing of the irradiated sample but avoiding foaming. The temperature of the cell was kept at 5–10 °C by circulating cold tap water. Samples were photolyzed for 2 min unless otherwise stated.

Irradiation of Liver Plasma Membranes, Glucagon, and Glucagon-NAPS. The irradiation effects on membranes, glucagon, and glucagon-NAPS were evaluated with the adenylate cyclase assay. Liver plasma membranes (0.5 mL of 0.5 mg of protein/mL) in 30 mM Tris-HCl, pH 7.0, containing 1 mg of BSA/mL or glucagon or glucagon-NAPS (10^{-6} M) in the same buffer (0.5 mL) with 1 mg of BSA/mL and 750 KIU/mL Trasylol were each separately irradiated for up to 2 min as described above. Membrane samples removed after varying times of irradiation were assayed for their adenylate cyclase activity in the presence and absence of 10^{-7} M glucagon (final membrane concentration 0.15 mg/mL). Irradiated peptide samples were tested at a final concentration of 10^{-7} M for their ability to stimulate adenylate cyclase in unphotolyzed liver plasma membranes. Adenylate cyclase assays were carried out under the standard assay conditions described above.

Photoaffinity Labeling of Liver Plasma Membranes by Glucagon-NAPS. Liver plasma membranes (1.5–2.0 mg/mL) were incubated with glucagon (10^{-8} M) or glucagon-NAPS (10^{-8} M) or were incubated in the absence of peptide hormone

in 0.5 mL of 30 mM Tris-HCl buffer, pH 7.0, containing 4 mg of BSA/mL and Trasylol (750 KIU/mL), at 30 °C, in the dark for 5 min. After incubation, the samples were precooled in ice for 3–5 min, saturated with N_2 gas for 1 min, and subsequently irradiated for 2 min under the conditions described above. Irradiation was followed by dilution of the membranes with 4 mL of 30 mM Tris-HCl buffer, pH 7.0, containing 10 mg of BSA/mL, 0.1 mM GTP, 1 mM EGTA, and 2 mM mercaptoethanol. The samples were incubated at 30 °C for 15 min to ensure dissociation of noncovalently bound hormone. The membranes were then pelleted by centrifugation (Sorval SS34 rotor, 19500*g*, 4 °C, 10 min). The supernatant was discarded, and the pelleted membranes were washed with 1.0 mL of 30 mM Tris-HCl buffer, pH 7.0. The membranes were subsequently resuspended in 30 mM Tris-HCl, pH 7, and 1 mg of BSA/mL and assayed for adenylate cyclase stimulation by glucagon, glucagon-NAPS, NaF, epinephrine, or isoproterenol. Adenylate cyclase assay conditions were as described above. Controls were performed by using membrane samples treated as above but not photolyzed as well as untreated membranes.

Binding Assay. Binding of glucagon and glucagon-NAPS to liver plasma membranes was assayed by displacement of radiolabeled peptide by assuming that the glucagon-specific receptor sites have an equivalent affinity for both native and iodinated glucagon or glucagon-NAPS. For binding studies in the absence of GTP and a nucleotide regenerating enzyme system, the incubation medium contained 1 mM EGTA, 0.2 mM cAMP, 10 mM $MgCl_2$, 2 mM mercaptoethanol, 1 mM aminophylline, 7 mM bacitracin, BSA (4 mg/mL), and Trasylol (750 KIU/mL) in 30 mM Tris-HCl, pH 7.0. For binding studies in the presence of 0.1 mM GTP, the incubation medium was similar to that of the adenylate cyclase assay with the addition of 750 KIU/mL Trasylol; [^{14}C]ATP was substituted with 1 mM unlabeled ATP. The final assay volume was 0.1 mL. The concentrations of the monoiodoglucagon and monoiodoglucagon-NAPS peptides, used as tracers, varied from assay to assay between 1.0×10^{-11} and 10.0×10^{-11} M [specific activity $(2.5–3.0) \times 10^6$ cpm/pmol]. Unlabeled or radiolabeled ligands added were in 30 mM Tris-HCl buffer, pH 7.0, containing 1 mg of BSA/mL and Trasylol (750 KIU/mL). After equilibration of the incubation medium at 30 °C for 1 min, the mixture was completed with the addition of liver plasma membranes suspended in 30 mM Tris-HCl, pH 7.0, containing 4 mg of BSA/mL to give a final membrane protein concentration of 0.08–0.10 mg/mL. After a 10-min incubation at 30 °C, membrane-bound ligand was separated from free ligand in a 90-μL sample of the incubation medium by the centrifugation method with a Beckman microfuge B as described by Rodbell et al. (1971a).

The time course of the binding of radiolabeled ligand to liver plasma membranes was measured. Membrane-bound ligand was separated from free ligand either by the centrifugation method or by filtration using glass filters (Whatman GF/B) which had been presoaked for 10 min in 30 mM Tris-HCl buffer, pH 7.0, containing 10 mg of BSA/mL. The filters were washed once with 0.5 mL of 2.5 mg of BSA/mL in 30 mM Tris-HCl, pH 7.0, before and after sample application. Nonspecific binding was defined as radioligand binding which took place in the presence of 10^{-6} M unlabeled glucagon. All binding assays were performed in Eppendorf tubes (1.5-mL capacity).

Determination of Glucagon Degradation by Liver Plasma Membranes. Glucagon (10^{-10}–10^{-6} M) and tracer ^{125}I-labeled glucagon were incubated in the binding assay media (±GTP) in the presence and absence of 0.10–1.0 mg of membrane protein/mL. After incubation at 30 °C for 10 min, 0.2 mL of an ice-cold solution of 10% (w/v) BSA in 30 mM Tris-HCl, pH 7.0, followed by 1.0 mL of 15% (w/v) ice-cold trichloroacetic acid, was added to all samples which were then left to stand on ice for 1 h. The precipitated protein was pelleted by centrifugation in the Beckman microfuge at 4 °C for 10 min (10000*g*). Aliquots of 1.0 mL of the supernatant were counted. The amount of degraded hormone was calculated from the increase in Cl_3CCOOH nonprecipitable counts with time in the presence of membrane.

Determination of the Dissociation of Membrane-Bound Hormone by 0.1 mM GTP after Photolysis. Liver plasma membranes, 1.25 mg in 0.5 mL of binding assay media (±GTP), were preincubated with ^{125}I-labeled glucagon-NAPS (10^{-10}–10^{-8} M) in the dark and subsequently photolyzed for 2 min as previously described. The amount of bound peptide was measured in 50-μL samples by the centrifugation method. The rest of the sample was diluted 8–10-fold with 30 mM Tris-HCl, pH 7.0, containing 0.1 mM GTP, 10 mg of BSA/mL, 1 mM EGTA, and 2 mM mercaptoethanol and subsequently incubated at 30 °C for 15 min. The membranes were then pelleted by centrifugation at 12500*g*, at 4 °C for 30 min. The supernatant was discarded, and the pellets were washed with 1.0 mL of 30 mM Tris-HCl, pH 7.0, and resuspended in the same buffer. The remaining amount of radiolabeled hormone bound was measured in 50-μL samples by the centrifugation method. Nonspecific binding was corrected for.

The amount of free peptide present in the incubation media (±GTP) was measured by determining the amount of peptide that would rebind to new membranes. ^{125}I-Labeled glucagon was incubated in 0.5 mL of binding assay medium (±GTP) as described under Binding Assay followed by centrifugation. Aliquots of the supernatants (0.1 mL) were tranferred into tubes containing 25 μL of the appropriate incubation medium. Fresh membrane was added to a final concentration of 0.10–0.70 mg of membrane protein/mL, and the samples were incubated at 30 °C for 10 min (final volume 0.15 mL). Membrane-bound peptide was separated from free peptide in 0.1-mL samples by the centrifugation method used in the binding studies. Nonspecific binding was subtracted.

Membrane Protein Determination. Membrane protein was determined by the modified mini-Lowry protein assay according to the method of Hess et al. (1978), with bovine serum albumin as standard.

Results

Adenylate Cyclase. Dose–response curves of adenylate cyclase stimulation by glucagon and glucagon-NAPS, in the presence of GTP, are presented in Figures 1 and 2, respectively. Half-maximal stimulation of the enzyme occurred at 1.60 nM glucagon (Figure 1) and at 0.45 nM glucagon-NAPS (Figure 2). Maximal stimulation by glucagon-NAPS was about 10% lower than that of native glucagon (Figure 2). These results are similar to those reported previously for this derivative (Demoliou & Epand, 1980). The quantitative differences may arise from the different conditions used such as the lowering of the pH from 7.6 to 7.0, raising the GTP concentration from 10 to 100 μM, and the introduction of 7 mM bacitracin in the present work. Maximal stimulation in the absence of GTP was only about 1–2-fold above the basal level irrespective of the peptide hormone used (data not shown). NaF (10 mM) stimulated adenylate cyclase about 6-fold above the basal rate.

The effects of the photolysis conditions on the adenylate cyclase assay were studied. Under the experimental conditions

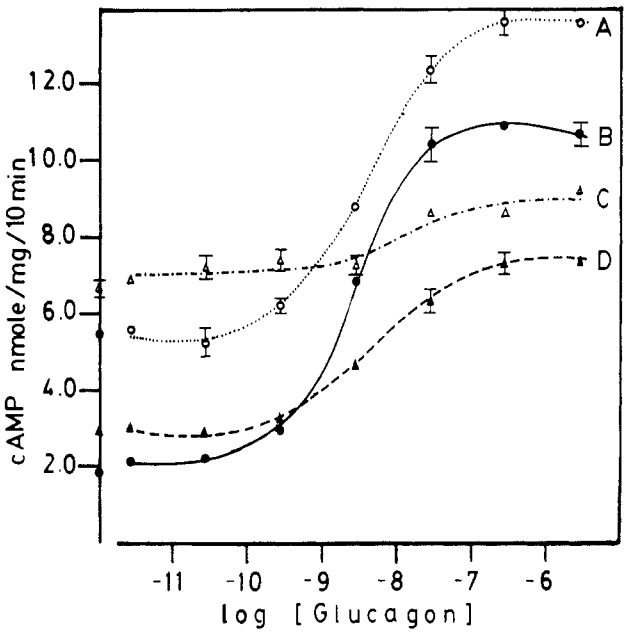

FIGURE 1: Glucagon dose–response curves of adenylate cyclase activity. Liver plasma membranes were assayed for enzyme stimulation by glucagon in the presence of 0.1 mM GTP. For the irradiation studies, membranes were preincubated with glucagon or glucagon-NAPS (10^{-8} M) and subsequently photolyzed or kept in the dark. Pretreated membranes were then diluted and incubated with 0.1 mM GTP for the dissociation of noncovalently bound hormone. After being centrifuged and washed, the resuspended membranes were assayed for adenylate cyclase stimulation by glucagon. Points and error bars represent the mean ± SEM of three or four parallel determinations of experiments repeated twice. (A, O) Membranes preincubated with glucagon in the dark; (B, ●) untreated membranes; (C, △) membranes preincubated with glucagon-NAPS, irradiated; (D, ▲) membranes preincubated with glucagon, irradiated.

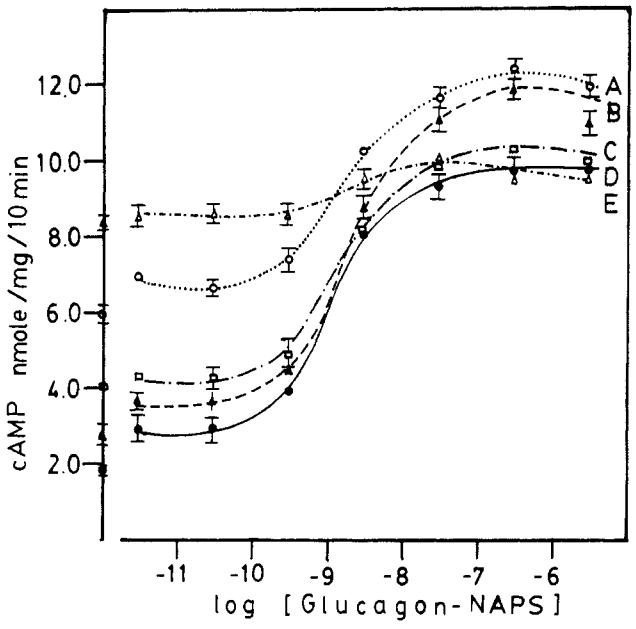

FIGURE 2: Glucagon-NAPS dose–response curves of adenylate cyclase activity. Liver plasma membranes were assayed for adenylate cyclase stimulation by glucagon-NAPS in the presence of 0.1 mM GTP. For the irradiation studies, membranes were treated as described in the legend to Figure 1 but assayed for adenylate cyclase stimulation by glucagon-NAPS. Points and error bars represent the mean ± SEM of three parallel determinations of experiments repeated twice. (A, O) Membrane preincubated with glucagon-NAPS in the dark; (B, ▲) membranes preincubated with buffer in the dark; (C, □) membrane preincubated with buffer, irradiated; (D, ●) untreated membranes; (E, △) membranes preincubated with glucagon-NAPS, irradiated.

Table I: Irradiation Effects on Rat Liver Plasma Membranes and on Glucagon and Glucagon-NAPS[a]

irradiation time (s)	adenylate cyclase % stimulation[b] membranes[c]	glucagon[d]	glucagon-NAPS[d]
0	100 ± 2	100 ± 2	100 ± 2
15	91 ± 5	108 ± 5	91 ± 2
30	93 ± 4	102 ± 6	91 ± 1
60	90 ± 7	101 ± 5	88 ± 2
90	84 ± 7	110 ± 6	84 ± 2
120	86 ± 4	101 ± 2	85 ± 2

[a] Basal activity of nonirradiated membranes was 3.0 ± 0.1 nmol mg^{-1} $(10\ min)^{-1}$. Maximal activity with nonirradiated hormone was 12.69 ± 0.03 and 8.4 ± 0.2 nmol mg^{-1} $(10\ min)^{-1}$ with glucagon and glucagon-NAPS, respectively. Values are the mean ± SEM of three determinations for membranes and glucagon and four determinations for glucagon-NAPS. Irradiation conditions are as described under Methods. [b] Stimulation is expressed as the percent of that obtained with nonirradiated peptide added to nonirradiated membranes. [c] Liver plasma membranes (0.53 mg of protein/mL) were irradiated. Samples removed at each time point were tested for adenylate cyclase stimulation by glucagon. [d] Glucagon and glucagon-NAPS (10^{-6} M) were irradiated. Samples removed at each time point were tested for adenylate cyclase stimulation at a final hormone concentration of 10^{-7} M.

used for the irradiation studies, more than 95% of glucagon-NAPS (10^{-4}–10^{-5} M in glacial acetic acid) was photolyzed within 2 min of irradiation ($t_{1/2} < 25$ s). This was determined by measuring the changes in the absorbance of the photolyzed sample at 395 nm, as previously reported (Demoliou & Epand, 1980). Irradiation of liver plasma membranes for an equivalent amount of time resulted in approximately 15% reduction of the adenylate cyclase response to glucagon stimulation (Table I). The decrease in the enzyme response was attributed to irradiation damage of the membranes since nonirradiated membranes, similarly treated in the dark, were not affected. Irradiation of glucagon did not affect the ability of the peptide to stimulate adenylate cyclase (Table I). Glucagon-NAPS, however, when irradiated stimulated adenylate cyclase less than the unphotolyzed peptide (Table I). Intra- and intermolecular cross-linking could contribute to this inactivation.

Dose–response curves of adenylate cyclase stimulation in plasma membranes irradiated in the presence of glucagon-NAPS are presented in Figures 1 and 2. The measured production of cAMP was linear with time for at least 20 min. The higher basal activities of membranes covalently labeled by glucagon-NAPS (Figures 1 and 2) than those irradiated in the presence of glucagon (Figure 1) suggest covalent labeling of receptor sites related to adenylate cyclase stimulation. The lower response of covalently labeled membranes to further stimulation of adenylate cyclase with increasing peptide concentrations (Figures 1 and 2) suggests an irreversibly activated enzyme. In addition to this effect, pretreatment of membranes with hormone or irradiation affected the response of the enzyme to further hormonal stimulation (Figures 1 and 2). An imcomplete dissociation of bound peptide, even after pretreatment with GTP, may account for the higher basal activities after pretreatment of membranes with hormone. However, even in this case, the adenylate cyclase could be further stimulated at higher hormone concentrations while this was not the case for the membranes photolyzed in the presence of glucagon-NAPS.

Specificity of covalent labeling of glucagon receptor sites by glucagon-NAPS was tested by measuring the adenylate cyclase response to subsequent stimulation by epinephrine and isoproterenol. Approximately the same stimulation was observed by either reagent in irradiated and nonirradiated

Table II: Adenylate Cyclase Activation in Liver Plasma Membranes Photolabeled Covalently with Glucagon-NAPS before Assay[a]

condition[c]	adenylate cyclase activity[b] [nmol (mg of protein)$^{-1}$ (10 min)$^{-1}$] basal	glucagon (10^{-7} M)	epinephrine (10^{-5} M)	isoproterenol (10^{-4} M)	NaF (10^{-2} M)
1	2.12 ± 0.06	15.0 ± 0.5	3.10 ± 0.06	2.83 ± 0.09	12.63 ± 0.13
2	1.21 ± 0.06	9.55 ± 0.4	1.95 ± 0.03	1.80 ± 0.05	8.64 ± 0.15
3	2.39 ± 0.09	10.5 ± 0.3	2.99 ± 0.03	2.8 ± 0.2	9.67 ± 0.14
4	0.98 ± 0.09	5.3 ± 0.1	1.23 ± 0.05	1.15 ± 0.03	6.21 ± 0.14
5	4.8 ± 0.2	7.0 ± 0.3	5.17 ± 0.06	5.26 ± 0.09	9.29 ± 0.05

[a] Liver plasma membranes (1.5 mg/mL) were preincubated with 10^{-8} M glucagon-NAPS or buffer and were subsequently irradiated or kept in the dark. After dissociation of noncovalently bound hormone by GTP (see legend to Figure 1), these membranes, at a final concentration of 0.08–0.20 mg/mL, were assayed for adenylate cyclase stimulation by glucagon, epinephrine, isoproterenol, and NaF. Results are mean ± SEM of four parallel determinations. [b] The adenylate cyclase assay was carried out under standard assay conditions with the addition of 0.01% (w/v) ascorbate to the incubation medium. [c] 1, Untreated (control) membranes; 2, membranes preincubated with buffer in the dark; 3, membranes preincubated with 10^{-8} M glucagon-NAPS, in the dark; 4, membranes preincubated with buffer, irradiated; 5, membranes preincubated with 10^{-8} M glucagon-NAPS, irradiated.

Table III: Equilibrium Dissociation Constants of Glucagon and Glucagon-NAPS Binding to Liver Plasma Membranes in the Presence and Absence of 0.1 mM GTP[a]

ligand	GTP	site	K_d (M)	binding capacity (pmol/mg)
glucagon	−	high affinity	$(3.5 \pm 0.7) \times 10^{-10}$	0.34 ± 0.15
		low affinity	$(4.0 \pm 1) \times 10^{-9}$	3.1 ± 0.6
	+	high affinity	$(6.0 \pm 1) \times 10^{-10}$	0.31 ± 0.11
glucagon-NAPS	−	high affinity	$(1.3 \pm 0.1) \times 10^{-10}$	0.67 ± 0.10
		low affinity	$(7.3 \pm 1.1) \times 10^{-10}$	2 ± 1
	+	high affinity	$(5 \pm 1) \times 10^{-10}$	0.7 ± 0.2

[a] Equilibrium constants were calculated from Scatchard plots by the method of Kahn et al. (1974). Briefly, low-affinity constants were obtained from the slope of a linear regression fit of the data for a concentration of 40–300 nM; the total number of binding sites was obtained from the intercept of this line on the abscissa. After the data obtained at a concentration less than 40 nM were corrected for the contribution of the low-affinity sites, they were also fitted by a linear regression analysis. The slope and the abscissa intercept of this line gave the high-affinity constant and the binding capacity. From the difference between total and high-affinity sites, a value for the binding capacity of the low-affinity site was obtained. In the presence of GTP, only the parameters for the high-affinity site were calculated. Values represent the mean ± SEM of three independent determinations.

membranes preincubated with glucagon-NAPS (Table II). The adenylate cyclase response to NaF stimulation in membranes covalently labeled with glucagon-NAPS was about 50% lower than the response of similarly treated nonirradiated membranes (Table II).

Hormone Binding to Membranes. The kinetic characteristics of ^{125}I-labeled glucagon-NAPS (10^{-11} M) binding to liver plasma membranes are illustrated in Figure 3. In the absence of GTP, steady state was reached in about 10–15 min. In the presence of the nucleotide, constant levels of bound hormone were reached in less than a minute (Figure 3B). The time course for ^{125}I-labeled glucagon binding was similar (data not shown). Binding of glucagon-NAPS to the glucagon receptor site was investigated by competition studies between ^{125}I-labeled glucagon or ^{125}I-labeled glucagon-NAPS and unlabeled glucagon or glucagon-NAPS (Figures 4 and 5). As illustrated, both peptides were able to displace more than 90% of bound hormone at concentrations $\geq 10^{-8}$ M. In the absence of GTP and at ligand concentrations of 0.3–0.5 nM, glucagon-NAPS displayed a higher affinity than glucagon. This apparent difference disappeared when GTP was included in the binding medium (Figures 4 and 5). In order to have a more quantitative comparison of the binding affinities of the two analogues, we analyzed the results according to the modified Scatchard method of Kahn et al. (1974). There was

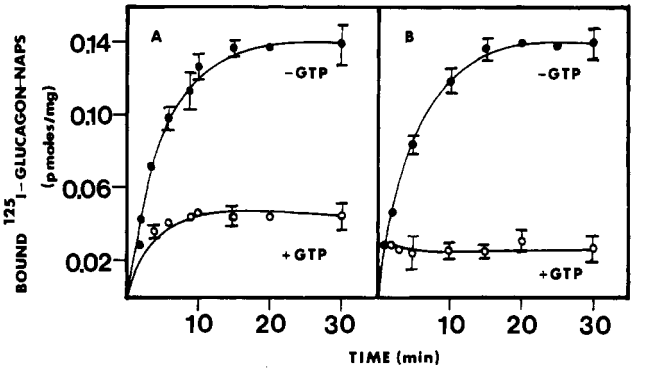

FIGURE 3: Time course of ^{125}I-labeled glucagon-NAPS binding to liver plasma membranes in the absence and presence of 0.1 mM GTP. Specific binding of ^{125}I-labeled glucagon-NAPS [$(2.0–5.0) \times 10^{-11}$ M, specific activity $(2.0–3.0) \times 10^6$ cpm/pmol] to plasma membranes (0.08–0.10 mg of protein/mL) was assayed as described under Methods. Bound from free hormone was separated by centrifugation (A) or by filtration (B). Error bars indicate the SEM of triplicate determinations in parallel except when they fall within the symbols.

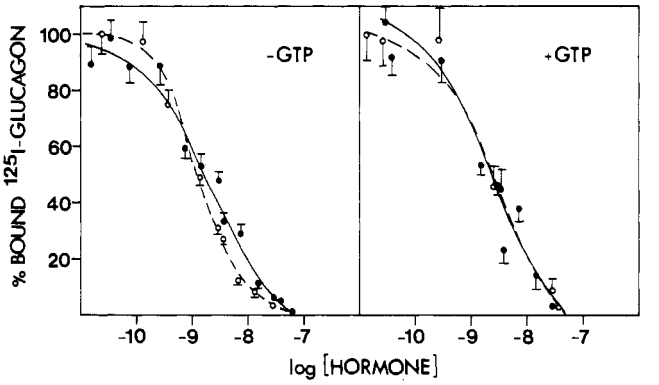

FIGURE 4: Displacement of ^{125}I-labeled glucagon by unlabeled glucagon and glucagon-NAPS. ^{125}I-Labeled glucagon [$(0.8–4.0 \times 10^{-11}$ M, specific activity $(2.5–3.0) \times 10^6$ cpm/pmol] was added to incubation media (±0.1 mM GTP) containing the indicated concentrations of unlabeled glucagon or glucagon-NAPS. Plasma membrane concentration was 0.08–0.10 mg of protein/mL in a total volume of 0.1 mL. After a 10-min incubation at 30 °C, bound from free hormone was separated by centrifugation. The amount of radiolabeled bound hormone was calculated as a percent of total amount bound in the absence of unlabeled peptide after correcting for nonspecific binding and hormone degradation. Points and error bars represent the mean ± SEM of three or four parallel determinations of two membrane preparations. (●) Glucagon; (○) glucagon-NAPS.

an indication of a small population of binding sites of higher affinity in the absence of GTP. When these were considered to be an independent class of binding sites, the results could be fitted well although there is a large error associated with the number of high-affinity binding sites (Table III). Analysis of the binding data according to Hill (1910) gave plots with

Table IV: Fraction of Free ^{125}I-Labeled Glucagon in the Binding Assay in the Presence and Absence of 0.1 mM GTP[a]

	preincubation with plasma membranes		preincubation without plasma membranes		
GTP	^{125}I-labeled glucagon (M)	specific binding (pmol/mg)	^{125}I-labeled glucagon (M)	specific binding (pmol/mg)	% free hormone
−	9.25×10^{-11}	0.110 ± 0.008	10.19×10^{-11}	0.18 ± 0.02	60 ± 2
+	9.67×10^{-11}	0.047 ± 0.004	10.55×10^{-11}	0.05 ± 0.007	93 ± 4

[a] ^{125}I-Labeled glucagon (10^{-10} M) was preincubated with or without plasma membranes (0.12 mg/mL) at 30 °C for 10 min. Free glucagon was separated from bound hormone and tested for rebinding to fresh membranes as described under Methods. Free hormone was calculated as a percentage of bound ^{125}I-labeled glucagon when preincubated in the absence of membranes, assuming the same specific activity (cpm/pmol) before and after incubation. Specific binding was calculated after correcting for nonspecific binding in the presence of 10^{-6} M unlabeled glucagon and for hormone degradation. Values are the mean ± SEM of three determinations in parallel.

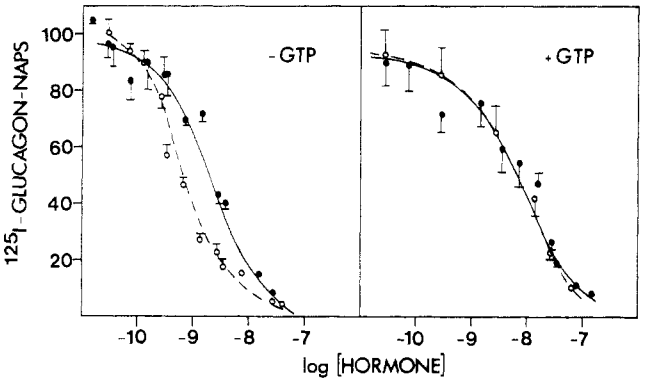

FIGURE 5: Displacement of ^{125}I-labeled glucagon-NAPS by unlabeled glucagon and glucagon-NAPS. The percent of ^{125}I-labeled glucagon-NAPS [(1.3–1.4) × 10^{-11} M, specific activity (2.8–3.0) × 10^6 cpm/pmol] specifically bound to plasma membranes in the presence of increasing concentrations of unlabeled glucagon and glucagon-NAPS was measured as described for ^{125}I-labeled glucagon in the legend to Figure 4. Points and error bars represent the mean ± SEM of three or four parallel determinations of two membrane preparations. (●) Glucagon; (○) glucagon-NAPS.

slopes of 1.15 ± 0.15 and 1.27 ± 0.12 for glucagon in the absence and presence of GTP, respectively, and 0.99 ± 0.10 and 1.01 ± 0.10 for glucagon-NAPS in the absence and presence of GTP, respectively, in the range 0.1–10 pmol of free peptide/mg of membrane protein. These values are indicative of noncooperativity. At higher peptide concentrations, the plots were curvilinear with slopes >1.0, suggesting positive cooperativity. As a result of the high specific activity of the monoiodinated peptides, a low concentration of radiolabeled ligand was used as tracer, leading to very low (2–5%) nonspecific binding. Binding was proportional to membrane concentrations in the region of 0.1–0.2 mg of membrane protein/mL, and at 0.10 mg of membrane protein/mL, degradation of ligand did not exceed 5% as measured by trichloroacetic acid precipitations. The dissociation of membrane-bound hormone by GTP (Rodbell et al., 1971b) was also shown by measuring the amount of free peptide present in the incubation medium of the binding assays (Table IV). Approximately 60% of the total peptide was found in the supernatant with membranes incubated in the absence of GTP, but more than 90% of the peptide was available for rebinding to new membranes in the presence of GTP.

Covalent Labeling of Plasma Membranes with ^{125}I-Labeled Glucagon-NAPS. Before dissociation by GTP, there is both covalent and noncovalent binding of glucagon-NAPS to plasma membranes (Table V). The decrease of the specifically bound peptide in photolyzed samples can be attributed to photolytic decomposition of iodine from the peptide as well as a lower affinity of glucagon-NAPS photolysis products for noncovalent binding to the membrane. Iodine cleavage during irradiation, under similar conditions, has been previously observed with ^{125}I-labeled glucagon (Demoliou & Epand, 1980). The amount

Table V: Covalent Labeling of Specific Receptor Sites in Liver Plasma Membranes by ^{125}I-Labeled Glucagon-NAPS after Irradiation[a]

	specific binding (pmol/mg)			
	before dissociation		after dissociation	
^{125}I-labeled glucagon-NAPS (M)	unphotolyzed	photolyzed	unphotolyzed	photolyzed
1.7×10^{-8}	2.4 ± 1.2	2.1 ± 0.7	0.05 ± 0.05	0.12 ± 0.08
1.7×10^{-9}	2.3 ± 0.7	1.4 ± 0.2	0.04 ± 0.03	0.10 ± 0.02
8.5×10^{-10}	1.7 ± 0.2	1.3 ± 0.1	0.04 ± 0.06	0.10 ± 0.01

[a] ^{125}I-Labeled glucagon-NAPS [specific activity (2.5–3.0) × 10^6 cpm/pmol] was incubated with liver plasma membranes (2.5 mg/mL) in the binding medium (−GTP). After irradiation, the amount of specifically bound hormone was measured (before dissociation), and the remaining sample was incubated with 0.1 mM GTP and the amount of specifically bound hormone again measured (after dissociation). Bound peptide was measured in pmol/mg of protein after correcting for nonspecific binding and peptide degradation. Results are the mean ± SEM of triplicate determinations.

of covalently bound peptide was measured after dissociation of noncovalently bound hormone by incubating the membranes with GTP. More than 90% of specifically bound peptide dissociated after incubation of the membrane with the nucleotide (Table V). Nonspecific binding to irradiated membranes ranged between 60 and 80% of bound peptide even after pretreatment with GTP, depending on the concentration of labeled ligand used. As a result, it was not possible to quantitate the extent of total covalent labeling of irradiated membranes. Specific covalent labeling of membranes which were incubated and irradiated in the presence of GTP could not be determined due to high nonspecific binding.

Discussion

One of the criteria for photoaffinity labeling of hormone receptor sites is that the photoactivatable derivative interacts with the receptors in the same fashion as the natural hormone. Photoaffinity probes have the advantage that these interactions can be studied in the dark, thereby avoiding any covalent cross-linking with the probe. In the case of glucagon-NAPS, we have shown that it behaves very similarly to glucagon with regard to interaction with specific rat liver plasma membrane receptor sites and subsequent activation of adenylate cyclase. The dose–response curve for the activation of adenylate cyclase by glucagon-NAPS (Figure 2) shows only small differences with that for glucagon (Figure 1), with the derivative having a slightly greater potency but a slightly lower maximal activation. In addition, maximal stimulation of adenylate cyclase by glucagon-NAPS had the same requirement for GTP as had been found for glucagon (Rodbell et al., 1971c). We have also compared the interaction of glucagon-NAPS with that of the native hormone by using a direct receptor binding assay. The results again indicate the similar behavior of the two peptides (Table III). These results are in agreement with studies of

other trytophan-modified derivatives of glucagon which showed little change in biological activity (Epand & Cote, 1976; Wright & Rodbell, 1980). It is possible that the small increase in potency for glucagon-NAPS results from increased hydrophobic interactions between the peptide and receptor or from the apparent altered conformational state of the peptide.[2]

We have also compared the binding of glucagon-NAPS to specific membrane receptor sites with that of glucagon. Glucagon-NAPS had a higher affinity than glucagon for the low-affinity sites in the absence of GTP (Table III). Two binding sites for glucagon have been observed with intact hepatocytes (Sonne et al., 1978) and with liver plasma membranes in the absence of GTP (Sperling et al., 1980). Some of the apparent binding heterogeneity may arise from the use of iodinated ligand if the affinities of labeled and unlabeled ligands are different. Differences have been observed between iodoglucagon and glucagon with respect to potency and affinity (Bromer et al., 1973; Desbuquois, 1975; Lin et al., 1976) partially due to the change in pK of the iodotyrosyl residues (Lin et al., 1976). This factor was minimized in our studies by using only the monoiodo derivatives and by performing both the adenylate cyclase and binding assays at pH 7.0 (20% of unionized ligand). This lower pH also minimizes photolytic decomposition of iodotyrosine (Demoliou & Epand, 1980). Binding studies with [^{3}H]glucagon (Lin et al., 1977) have shown the same time dependence for steady-state binding in the presence and absence of GTP and gave binding isotherm curves similar to those observed in the present studies with monoiodinated peptides. These results support the conclusion that the glucagon binding sites are heterogeneous.

The results of photolysis studies of glucagon-NAPS with the rat liver plasma membrane show that this derivative not only binds to specific glucagon receptor sites but also reacts with them upon photolysis, leading to irreversible activation of adenylate cyclase. If this labeling were to the active form of the glucagon receptor, it would explain the ability of a small percentage of the receptor sites to activate adenylate cyclase. This finding could also be explained by a collision–coupling model analogous to the one proposed by Levitzki (1981) for the β-adrenergic receptor.

The results of the present studies have shown that glucagon-NAPS is similar to glucagon in its ability to bind to specific membrane receptor sites and to activate adenylate cyclase. It can therefore be used effectively for photoaffinity labeling of the glucagon receptor and its further characterization. Photoaffinity labeling of the glucagon receptor is specific and results in an irreversibly stimulated adenylate cyclase enzyme.

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Identification of the Glucagon Receptor by Covalent Labeling with a Radiolabeled Photoreactive Glucagon Analogue†

Catherine Demoliou-Mason and Richard M. Epand*

ABSTRACT: The photoreactive ^{125}I-labeled glucagon-NAPS [^{125}I-labeled 2-[(2-nitro-4-azidophenyl)sulfenyl]-Trp^{25}-glucagon] was used to label the glucagon receptor sites in rat liver plasma membranes. The proteins labeled were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis with or without reduction with dithiothreitol. The photoaffinity peptide specifically labeled a number of bands with apparent molecular weights >200 000 and probably at least two protein bands in the molecular weight range 52 000–70 000. The relative amounts of radioactivity associated with these bands and their relative mobilities differed in samples from reduced and unreduced membranes. Their relative mobilities also differed with percent acrylamide cross-linking, suggesting a glycoprotein nature and the presence of intramolecular disulfide bonds. A nonspecifically labeled band with an apparent molecular weight of 27 000–28 000 also displayed a similar behavior. Photolabeling in the presence of 0.1 mM guanosine 5′-triphosphate (GTP) decreased the amount of radiolabeling of these bands, suggesting their involvement in the glucagon stimulation of adenylate cyclase. The photolabeled receptor in the membranes, solubilized with Lubrol-PX and fractionated on an Ultrogel AcA22 column, eluted with an apparent molecular weight of 200 000–250 000. Addition of GTP to the solubilized glucagon receptor of nonirradiated membranes caused complete dissociation of the complex. Gel electrophoresis of the partially purified radiolabeled receptor identified the same protein components observed in photolabeled membranes. These results indicate that the glucagon receptor is an oligomer probably composed of at least two different subunits that are linked together or greatly stabilized by disulfide bonds. They also show that ^{125}I-labeled glucagon-NAPS can be used effectively to covalently label the putative glucagon receptor and thus aid in its further characterization.

Several studies have shown that the first step in the action of many hormones is binding to specific sites on target cell membranes containing adenylate cyclase systems responding to these hormones [see, for example, Rodbell (1980)]. The binding properties of glucagon receptor(s) in rat liver plasma membranes (Rodbell et al., 1971, 1974; Lin et al., 1977; Sperling et al., 1980), crude liver microsomal membranes (Desbuquois et al., 1974), intact hepatocytes (Sonne et al., 1978), and adipocytes (Birnbaumer & Pohl, 1973) have been extensively studied, but very little is known about the identity and character of the glucagon receptor itself. Initial attempts to characterize the glucagon receptor in Lubrol-PX-solubilized liver plasma membranes by gel fractionation have identified a macromolecule with a molecular weight of 190 000 (Blecher et al., 1974). Similar studies with the myocardial adenylate cyclase–glucagon complex have identified two components, one having a catalytic activity and a molecular weight greater than 100 000 and the other, a glucagon-binding fraction with a molecular weight of 24 000–28 000 (Levey, 1975). Active glucagon–agarose preparations have also been studied for their possible use in affinity chromatography (Krug et al., 1971; Johnson et al., 1972). Labeling of rat liver plasma membranes with radioactively labeled iodoacetamide or iodoacetic acid in the presence and absence of glucagon has identified a 240 000 molecular weight protein by sodium dodecyl sulfate ($NaDodSO_4$)[1]–polyacrylamide gel electrophoresis as being an integral component of the adenylate cyclase–glucagon receptor complex (Storm & Chase, 1975). Photoaffinity labeling of the glucagon receptor with the inactive derivative ^{125}I-labeled N^{ϵ}-(4-azido-2-nitrophenyl)-Lys^{12}-glucagon, which was able to bind to liver plasma membranes, has identified two components with a molecular weight range of 23 000–25 000 as the receptor sites for glucagon (Bregman & Levy, 1977). Recently, using photoaffinity cross-linking with hydroxysuccinimidyl *p*-azidobenzoate, Johnson et al. (1981) have identified an ^{125}I-labeled glucagon-binding membrane protein having a molecular weight of 53 000.

In this study, we have used a photoreactive radiolabeled glucagon derivative, ^{125}I-labeled 2-[(2-nitro-4-azidophenyl)-sulfenyl]-Trp^{25}-glucagon (^{125}I-labeled glucagon-NAPS)(Demoliou & Epand, 1980), to covalently label the glucagon receptor(s). The criteria and advantages of using the pho-

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[1] Abbreviations: ^{125}I-labeled glucagon-NAPS, ^{125}I-labeled 2-[(2-nitro-4-azidophenyl)sulfenyl]-Trp^{25}-glucagon; BSA, bovine serum albumin; App(NH)p, adenosine 5′-(β,γ-imidotriphosphate); cAMP, adenosine cyclic 3′,5′-phosphate; GTP, guanosine 5′-triphosphate; Gpp(NH)p, guanosine 5′-(β,γ-imidotriphosphate); Tes, 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; DNP-glycine, *N*-(2,4-dinitrophenyl)glycine; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid; KIU, kallikrein inactivator units; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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