EXHIBIT Q

Biochemistry, 1984, 23, 6191–6195

Functional Properties of Carbohydrate-Depleted Tissue Plasminogen Activator†

Sheila P. Little,* Nils U. Bang, Cathy S. Harms, Carole A. Marks, and Lawrence E. Mattler

ABSTRACT: In order to evaluate the importance of the carbohydrate moiety of human tissue plasminogen activator (TPA), human melanoma (Bowes) cells were treated with a glycosylation inhibitor, tunicamycin (TM), and cellular fractions were assayed for fibrinolytic activity. Where glycosylation was inhibited by 90% and protein synthesis by 30%, TPA specific activity measured by fibrinolytic assays decreased 6–10-fold in the tissue culture medium and cell cytosol with a concomitant 2-fold increase in the 100000*g* microsomal pellet. In addition, TPA purified to apparent homogeneity was treated with endo-β-*N*-acetylglucosaminidase H (Endo-H), producing a fraction that in contrast to native TPA did not adsorb to concanavalin A–Sepharose (Con A–Sepharose). This fraction represented TPA from which 85–90% of N-linked carbohydrate residues had been removed. Native TPA effectively activated plasminogen in the presence of fibrin (K_m = 1 μM, k_{cat} = 0.09 s^{-1}) whereas saturation of the enzyme was not achieved at 100 μM plasminogen in the absence of fibrin. Glycosidase-treated and native TPA activated plasminogen at identical high rates in the presence and at identical negligible rates in the absence of fibrin. These studies indicate that the inhibition of glycosylation of TPA results in the inhibition of secretion of the molecule as has been observed for some other glycoproteins. The enzymatic removal of N-linked carbohydrate from purified TPA does not change its unique fibrin-directed properties.

The retention of biological activity by glycoproteins void of carbohydrate is variable and unpredictable. In some instances, the absence of carbohydrate results in no loss of functional activity as is the case of the antiviral activity associated with α- and β-interferons (Kelker et al., 1983; Knight & Fahey, 1982). In other cases, murine C4 loses hemolytic activity (Karp et al., 1982), the ability of human chorionic gonadotropin to enhance adenylyl cyclase activity is impaired (Keutmann et al., 1983), or the von Willebrand–VIIIC complex appears inactive upon partial deglycosylation (Gralnick et al., 1983).

A recent study of the functional properties of deglycosylated monoclonal antibodies demonstrated defects located in the Fc domain of the molecule. Although deglycosylated monoclonal antibodies were capable of recognizing antigen, they were incapable of activating complement, did not bind to Fc receptors, and did not produce antibody-dependent cellular toxicity. These deglycosylated IgG molecules interestingly displayed a prolonged rather than shortened $t_{1/2}$ (Nose & Wigzell, 1983).

Tissue plasminogen activator (TPA)[1] is a protein containing approximately 6.8% (w/w) carbohydrate (Rånby et al., 1982). TPA enzymatically converts plasminogen into plasmin only at the site of the fibrin clot. Because it activates only fibrin-associated plasminogen, it is expected to be a useful thrombolytic agent and has been expressed in *Escherichia coli* as a recombinant DNA product (Pennica et al., 1983). The recombinant molecule contains no carbohydrate and is fibrinolytically active. However, it is not known whether the lack of the carbohydrate moiety affects the specific activity of TPA or the kinetics of plasminogen activation. We have sought to examine the functional properties of TPA depleted of carbohydrate by in vivo and in vitro approaches to better assess the potential problems associated with recombinant-derived TPA.

Materials and Methods

Materials

Urokinase (*UK*) was Abbott reference UK (lot no. 2021-209) containing 2100 IU/mL or WHO standard UK (4800 IU/vial).

Human plasminogen was purified from human plasma by lysine–Sepharose chromatography (Deutsch & Mertz, 1970). The resultant preparations possessed specific activities of 21–24 IU/mg and contained 10 ppm of plasmin. In some experiments plasmin activity was eliminated through overnight incubation with 1 mM phenylmethanesulfonyl fluoride.

Bovine fibrinogen (Miles Laboratories) was rendered plasminogen free by lysine–Sepharose chromatography (Deutsch & Mertz, 1970).

Fibrin monomer was prepared from human fibrinogen (Kabi, grade L) in 0.05 M phosphate, 0.3 M NaCl, and 0.01 M EDTA, pH 7.3 (buffer A), clotted in a 100 mm diameter Petri dish with human thrombin (1 unit/mg of fibrinogen). The resultant clot was collected on a nylon stocking filter and washed extensively with buffer A in a Büchner funnel. The clot was redissolved in 0.2 M sodium acetate, 2 M KBr, and 0.01 M EDTA, pH 5.5. The clot was repolymerized through 20-fold dilution in 0.1 M phosphate buffer, pH 7.3 (buffer C), washed extensively in buffer C, and redissolved in buffer B to a final protein concentration of 0.6 mg/mL. The monomer preparation was kept frozen in aliquots at −70 °C until use.

D-[^{14}C]Glucosamine, [^{3}H]leucine, and [^{3}H]mannose were purchased from New England Nuclear.

Endo-H was purchased from Miles Laboratories.

Plasminogen-free human thrombin (specific activity 2800 NIH units/mg) was a generous gift from Dr. J. W. Fenton II, Albany, NY.

Growth of Cells. An established human melanoma cell line (Bowes) was obtained from Dr. D. B. Rifkin, Rockefeller

† From the Molecular Biology Department, Lilly Research Laboratories, Indianapolis, Indiana 46285 (S.P.L.), and the Hematology Services Department, Lilly Research Laboratories, and the Departments of Medicine and Pathology, Indiana University School of Medicine, Indianapolis, Indiana 46202 (N.U.B., C.S.H., C.A.M., and L.E.M.).
Received March 22, 1984.

[1] Abbreviations: TPA, tissue plasminogen activator; IU, international units; WHO, World Health Organization; TM, tunicamycin; Endo-H, endo-β-*N*-acetylglucosaminidase H; GlcNAc, *N*-acetylglucosamine; Con A–Sepharose, concanavalin A–Sepharose; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/84/0423-6191$01.50/0

University, New York. Cells were maintained in Eagle's essential medium supplemented with 10% newborn calf serum at 37 °C in a 5% CO_2 humidified atmosphere (Rijken & Collen, 1981). For experiments involving the treatment of cells with TM, confluent monolayers of Bowes cells were prepared in 100-mm Petri dishes.

TPA was purified from conditioned media of the Bowes human melanoma cell line essentially according to Rijken & Collen (1981) or by immunoabsorption (see below). The purified materials possessed specific activities of 10^5 units/mg by standard fibrin plate assay (see below). The materials appeared homogeneous by SDS–polyacrylamide gel electrophoresis under nonreducing conditions (apparent M_r 68 000); under reducing conditions, the purified enzyme preparations consisted of roughly equal parts of one-chain and two-chain TPA (apparent M_r of chains 32 000 and 37 000).

All other materials and reagents used were of analytical grade.

Methods

Preparation of Rabbit Anti-TPA Antisera and Sepharose Affinity Chromatography. Antisera against purified TPA were raised in rabbits by injecting 100 μg of purified protein emulsified in Freund's complete adjuvant. Injections were given at 3-week intervals.

The IgG fraction of antisera was isolated by affinity chromatogaphy on protein A–Sepharose (Pharmacia) with desorption of antibodies using 1 M acetic acid collecting 0.5-mL fractions into 0.5 mL of 0.5 M Tris base. Anti-TPA IgG was conjugated for affinity chromatography with activated CNBr–Sepharose 4B.

Plasminogen Activator Assays. Four types of plasminogen activator assays were utilized. (1) One was the amidolytic activity assay using the chromogenic substrate S2288 (H-D-Ile-Pro-Arg-pNA·2HCl) (Kabi) according to the recommendations by the manufacturers with certain modifications. Briefly, TPA-containing samples (0.01–0.2 mL) (appropriately diluted in assay buffer) were mixed with assay buffer (0.05 M Tris, 0.15 M NaCl, 0.01% Tween 80, pH 8.8) with Trasylol added to 10 units/mL (aprotinin, FBA Pharmaceuticals) and 0.05 mL of 3 mM S2288. The rate of hydrolysis of S2288 was measured as the ΔA_{405nm}/min and at 37 °C in a Beckman Model 35 recording spectrophotometer. The kinetic data for this substrate using urokinase and TPA were close (urokinase, K_m = 0.22 mM, k_{cat} = 19 s^{-1}; TPA, K_m = 0.24 mM, k_{cat} = 24 s^{-1}). Therefore, it was considered reasonable to equate 1 TPA amidolytic unit to 1 WHO reference standard international urokinase unit. (2) Another was a standard fibrin plate assay with WHO urokinase as the reference standard (Haverkate & Brakman, 1975). (3) Another was the ^{125}I-labeled fibrin film assay [Unkeless et al. (1973) as modified by Hoylaerts et al. (1982)] utilizing bovine plasminogen free fibrinogen and human plasminogen free thrombin with 0.05 IU of plasminogen added to individual 16-mm microtiter wells (Costar cluster plates). (4) Another was the plasminogen activation rate assay in the absence and presence of fibrin monomer essentially according to Rånby & Wallén (1981) utilizing the chromogenic substrate S2251 (H-D-Val-Leu-Lys-pNA·2HCl) (Kabi). In assays performed in the presence of fibrin, fibrin monomer stock was added to 20 μL (total reaction mixture volume 1 mL).

Kinetic experiments were performed with 1 unit of TPA, 0–100 μM plasminogen, and 0.3 mM S2251 in 0.02 M NaH_2PO_4, 0.1 M NaCl, 10 mM EDTA, and 0.02% Tween 80, pH 7.3, and incubation times of 60 and 90 min at room temperature. Kinetic constants were calculated in the conventional Lineweaver–Burk transformation.

Protein in cell extracts was estimated by A_{280nm}/A_{260nm} measurements using Kalckar's equation (Kalckar, 1947). Protein in purified TPA preparations was estimated by A_{280nm} measurements using an extinction coefficient of 28.1 established by amino acid composition analysis.

Preparations of Carbohydrate-Depleted TPA. TPA depleted of carbohydrate in vivo was prepared by incubating Bowes cells in the presence of TM (lot 361-913-165A, Eli Lilly and Co.) at concentrations of 10 and 50 μg/mL. TM specifically blocks N-glycosylation of asparagine residues by inhibiting the synthesis of the oligosaccharide donor *N*-acetylglucosaminylpyrophosphoryldolichol (Takatsuki et al., 1975). TM was first dissolved in dimethyl sulfoxide (Me_2SO) before the drug was added to serum-free medium. The final Me_2SO concentration was 1 μL/mL. After an 18-h incubation at 37 °C, conditioned medium was removed and cells were processed essentially as described by Loskutoff & Edgington (1981). Cell monolayers were washed with cold phosphate-buffered saline and removed from the plastic surface with a rubber policeman. Cells were washed again by low-speed centrifugation at 800*g* for 5 min and resuspended in a hypotonic homogenization buffer (0.25 M sucrose, 0.1 mM EDTA, 0.01 M Tris-HCl, pH 8.1) at 4 °C. Cell pellets were homogenized in a tight-fitting glass Dounce homogenizer using 25–30 strokes to disrupt 90–95% of the cells. The nuclei were separated by centrifugation at 600*g* for 10 min. The cytoplasm was further fractionated into a particulate microsomal-rich fraction (100000*g* for 60 min) and cytosol supernatant. The microsomal pellets were extracted through repeated freezing and thawing in 0.14 M imidazole, 2 M KSCN, and 0.01% Tween 80, pH 7.5. Fibrinolytic activity was estimated in pellet extracts, cytosols, and conditioned media by the fibrin plate assay after overnight dialysis against 1 M $NaHCO_3$. To establish the degree of inhibition of glycosylation and protein synthesis, confluent monolayers of both cells were incubated for 18 h at 37 °C in the presence of TM at concentrations of 0, 10, 50, and 100 μg/mL in serum-free media after the addition of TM, D-[^{14}C]glucosamine (1 μCi/mL), and [^3H]leucine (1 μCi/mL). After 18 h, cells were harvested and washed and lysates prepared as above. Aliquots from each lysate were precipitated with equal volumes of 10% trichloroacetic acid and counted.

Deglycosylation of TPA was achieved in vitro by treating purified TPA with Endo-H. Endo-H causes the release of N-linked high-mannose oligosaccharides (Hubbard & Ivatt, 1981) and cleaves between the two proximal GlcNAc residues. Purified labeled TPA (60 μg) was incubated for 6 or 18 h at 37 °C with 0.1 unit of Endo-H in 0.1 M citrate, 0.01% Tween 80, and 0.1% SDS, pH 5.5. Another 60-μg TPA aliquot was incubated in the same buffer system without Endo-H. After dialysis against 0.15 M imidazole, 1 M NaCl, 0.5 M KSCN, and 0.01% Tween 80, pH 7.6, untreated and endo-H-treated TPA were applied to 0.2-mL Con A–Sepharose columns (Pharmacia). Columns were desorbed with 0.5 M methyl α-mannoside, 0.01 M phosphate, 2 M KSCN, and 0.01% Tween 80, pH 7.5. In two series of experiments, Bowes melanoma cells were exposed overnight to 3.6 μCi of [^{14}C]-glucosamine/mL and 36 μCi of [^3H]mannose/mL. Radiolabeled TPA was immunopurified from conditioned media, treated with Endo-H, and applied to Con A–Sepharose columns as above.

Electrophoresis. SDS–polyacrylamide gel electrophoresis was performed essentially according to Weber & Osborn (1969) under nonreducing or reducing conditions, in some

Table I: TPA Activity of Cell Fractions of Bowes Cells Treated with Tunicamycin[a]

TM concn (μg/mL)	100000g cell pellet (units/mg of protein)	100000g cell pellet supernatant (units/mg of protein)	conditioned media (units/mg of protein)
0	66.4	12.3	250.9
10	118.4	2.2	40.9
50	108.0	4.0	21.0

[a] Bowes cells were incubated for 18 h as described in Figure 1 in the presence of 0, 10, and 50 μg of tunicamycin. Harvested cells were distributed and separated into 100000g pellets and supernatants; 100000g pellets were extracted as described under Materials and Methods. Protein concentration in the 100000g pellet, 100000g pellet supernatant, and conditioned media was estimated by A_{280nm}/A_{260nm} measurements using Kalckar's equation (Kalckar, 1947). TPA activity was assayed in each fraction by the standard fibrin plate technique.

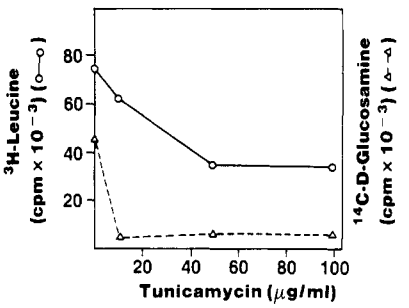

FIGURE 1: Influence of tunicamycin on incorporation of [^{14}C]-glucosamine and [^{3}H]leucine into Bowes cell trichloroacetic acid precipitable protein. Confluent monolayers of human melanoma (Bowes) cells were incubated as described under Materials and Methods.

experiments, as modified by Booyse et al. (1976), to allow solubilization for liquid scintillation counting. To assess the M_r distribution of fibrinolytic activity, cylindrical gels were soaked for 2 × 45 min in 2.5% Triton X-100 to remove SDS (Levin & Loskutoff, 1982), frozen on dry ice, and cut into 2-mm slices, which were placed on standard fibrin plates. The diameters of zones of lysis surrounding the slices were measured after overnight incubation at 37 °C.

Results

Isotopically Labeled Bowes Cells. When monolayers of Bowes melanoma cells were allowed to incubate in the presence of TM, the uptake of D-[^{14}C]glucosamine into glycoproteins, UDP-glucosamine, and other products was inhibited by 90% at a concentration of 10 μg/mL (Figure 1). Concomitantly, the uptake of [^{3}H]leucine was inhibited by 30%.

Cells treated with TM at concentrations of 10 and 50 μg/mL secreted into the media 6–10-fold less TPA as shown in a representative experiment seen in Table I. Cytosolic TPA also decreased in a similar manner. At the same time TPA activity in the microsomal fraction increased almost 2-fold at TM concentrations of 10 and 50 μg/mL.

When microsomal extracts from cells previously treated with TM, 10 μg/mL, were incubated with monospecific polyclonal TPA antibody, fibrinolytic activity as quantified in the ^{125}I-labeled fibrin film assay was inhibited 100% (data not shown).

Functional Properties of Carbohydrate-Depleted TPA. Endo-H treatment of purified TPA for 6 h yielded two species, peak 1 and peak 2, as exemplified by the Con A–Sepharose chromatogram displayed in Figure 2. Exhaustive digestion of TPA with Endo-H for 18 h yielded a similar pattern (data not shown). It appears that approximately 50% of the TPA preparation is resistant to Endo-H treatment. Authentic glycosylated TPA incubated with SDS-containing citrate buffer, pH 5.5, without Endo-H adsorbed almost 100% to Con A–Sepharose. The specific activities of three enzymatically deglycosylated Con A–Sepharose-isolated TPA peak 1 preparations were 97 000, 101 000, and 106 000 units/mg, no different from several batches of authentic glycosylated TPA.

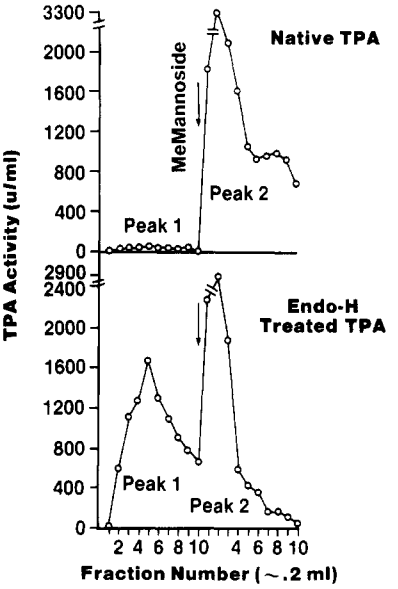

FIGURE 2: Separation of native and Endo-H-treated TPA on Con A–Sepharose. Purified TPA (60 μg) was incubated for 6 h at 37 °C with 0.1 unit of Endo-H as described under Materials and Methods. Another TPA aliquot was incubated in the same buffer without Endo-H. The dialyzed materials were applied to and desorbed from Con A–Sepharose as described under Methods. Fibrinolytic activity was quantified in the amidolytic S2288 assay.

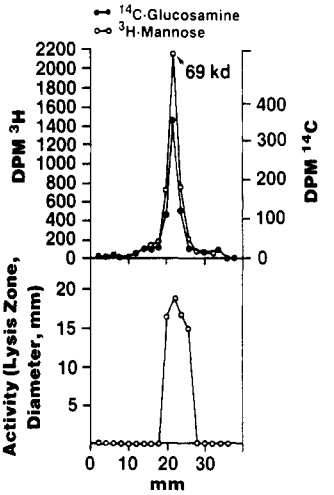

FIGURE 3: SDS–polyacrylamide gel electrophoresis (nonreducing conditions) of immunopurified TPA incorporating [^{14}C]glucosamine and [^{3}H]mannose: (upper panel) distribution of ^{14}C and ^{3}H radiolabels; (lower panel) distribution of fibrinolytic (fibrin plate) activity.

In order to determine the percentage of carbohydrate removed from native TPA by Endo-H treatment, Bowes melanoma cells were incubated with [^{3}H]mannose and [^{14}C]-glucosamine overnight. Radiolabeled TPA from the conditioned tissue culture media from these cells was purified by immunoabsorption. SDS–polyacrylamide gel electrophoresis demonstrated that the [^{14}C]glucosamine label and the [^{3}H]-

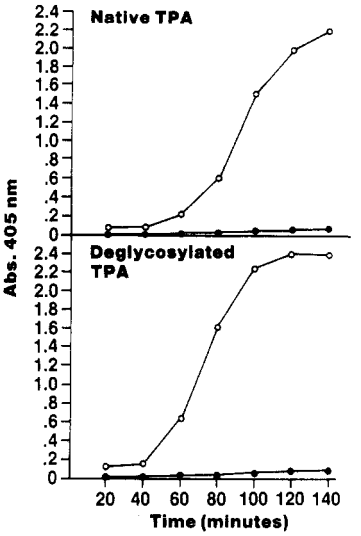

FIGURE 4: Rates of plasminogen activation by native and deglycosylated TPA in the presence and absence of fibrin. Native or deglycosylated TPA (1 IU/mL) (peak 1 material, Figure 2) was incubated with plasminogen (0.5 IU/mL) at room temperature with (open circles) or without (closed circles) fibrin monomer (20 μg/mL). Plasmin generation was quantified in an amidolytic assay (S2251, H-D-Val-Leu-Lys-pNA·2HCl).

Table II: Kinetics of Plasminogen Activation by TPA in the Presence and Absence of Fibrin

	K_m (μM Plg)	k_{cat} (s^{-1})	k_{cat}/K_m (nM^{-1} s^{-1})
fibrin	1.0 ± 0.02[a]	0.09 ± 0.02	90.0
no fibrin	100	ND	0.36

[a] Mean ± SEM; n = 3. In one experiment using enzymatically deglycosylated TPA in the presence of fibrin monomer, the K_m was 0.95 μM and the k_{cat}, 0.093 s^{-1}.

mannose label of this authentic immunopurified TPA comigrated in a sharp peak (M_r 67 000) and coincided with peak plasminogen activator activity (Figure 3). Endo-H digestion, followed by Con A–Sepharose chromatography as outlined above, resulted in the loss of 87% of the [^{14}C]glucosamine and 86% of the [^{3}H]mannose label from the peak 1 portion of this material.

Deglycosylated TPA peak 1 material was used in the assay, which reflects the rate of plasminogen activation in the presence and absence of fibrin. As noted in Figure 4, similar rapid rates of plasminogen activation in the presence of fibrin and similar negligible rates of activation in the absence of fibrin were noted for authentic and deglycosylated TPA. The kinetics of plasminogen activation by native TPA is given in Table II. These data are very similar to figures reported by Rijken et al. (1982). Like these authors, when examining plasminogen activation in the absence of fibrin monomer over plasminogen concentrations of 1–100 μM, we failed to saturate the enzyme at 100 μM. In calculating the catalytic efficiency (k_{cat}/K_m), we assumed, as did Rijken et al. (1982), that the reaction in the absence of fibrin obeyed Michaelis–Menten kinetics. Almost identical kinetic constants for plasminogen activation were achieved with enzymatically deglycosylated TPA.

Discussion

Our results indicate that TPA extensively depleted of carbohydrate either through TM treatment of the cell source or through enzymatic treatment of the purified protein retains biological fibrinolytic activity. In the case of Endo-H deglycosylated TPA, we were able to demonstrate unequivocally that this material retains the unique fibrin-directed properties of authentic TPA, i.e., the ability to activate plasminogen only in the presence of fibrin. Experiments in which glycosylation was inhibited in vivo by TM have some interpretative problems. We as well as others (Struck & Lennarz, 1977) have noted that the concentrations of TM required to effectively inhibit glycosylation also significantly reduce protein synthesis. In our hands, 10 μg/mL TM, while inhibiting glycosylation approximately 90%, inhibited protein synthesis by approximately 30%. Other investigators (Norrild & Pedersen, 1982; Brooks, 1983) achieved similar effects with 5–10-fold less TM, underscoring that different batches of TM vary considerably in potency.

Studies involving viral glycoproteins have suggested that carbohydrate residues may be important in the transport to the plasma membrane and for viral infectivity (Leavitt et al., 1977). Other studies have demonstrated that TM treatment prevents secretion of IgA and IgE but not IgG secretion from myeloma tumor cell lines (Hickman et al., 1977).

Our data suggest that inhibition of glycosylation retains TPA inside the microsomes, which contain the enzymatic apparatus necessary for glycosylation. Evidence has been presented by Hickman et al. (1977) suggesting from morphological data that nonglycosylated IgA is found associated with the rough endoplasmic reticulum. However, to our knowledge, accumulation of nonglycosylated protein in microsomal fractions has not been quantitatively demonstrated in the past. The significant increase in TPA activity in the 100000g pellets with a concomitant decrease in TPA activity in the cytosol and in the conditioned media in our study is best explained through this mechanism. Alternatively, the possibility cannot be excluded that secreted carbohydrate-deficient TPA is more susceptible to proteolytic degradation.

Enzymatic deglycosylation of TPA performed under mildly denaturing conditions failed to completely remove carbohydrate. Reported amino acid sequences for TPA (Pennica et al., 1983) indicate four potential sites for N-glycosidic linkage (sequences Asn-X-Ser or Asn-X-Thr). Three of these glycosidation sites are located in the "kringle" regions of the heavy chain of the TPA molecule thought to be essential for fibrin binding, and one potential glycosylation site is located in the serine protease portion of the molecule close to the active serine. One of the three potential glycosylation sites in the kringle regions probably is not functional since the amino acid intervening between Asn and Ser is Pro. It is not entirely surprising that half of the TPA treated with Endo-H was resistant to such treatment as evidenced by its persistent ability to absorb to Con A–Sepharose. We as well as others (Levin & Loskutoff, 1982; Berger & Tuttle, 1982) have evidence that this highly folded molecule containing 35 cysteine residues is highly resistant to denaturation in SDS, urea, and guanidine hydrochloride. It is reasonable to speculate that TPA molecules during treatment with Endo-H were not all in the denatured conformation and therefore not accessible to endoglycosidase digestion.

Knowing that TPA essentially void of carbohydrate retains the fibrin-dependent properties allows one to speculate on other problems that may be addressed by examining recombinant-derived nonglycosylated TPA. To date, the specific activity of the TPA expressed in *E. coli* has not been documented. Our study suggests that if the TPA molecule is allowed to form in the native conformation without carbohydrate, the specific activity should be similar to native TPA.

It has been demonstrated that terminal sialic acid and galactose residues are important in determining the hepatic clearance and plasma half-life of some glycoproteins (Zahlten

et al., 1981). How nonglycosylated TPA will react as an antigen, its biological half-life, and its interaction with extracellular inhibitors await further study.

Acknowledgments

The expert technical assistance of Jenna Walls is gratefully acknowledged.

Registry No. Plasminogen, 9001-91-6.

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Selective Nitration of Tyrosines-26 and -64 in Bacteriorhodopsin with Tetranitromethane†

Peter Scherrer* and Walther Stoeckenius

ABSTRACT: Nitration of tyrosine-26 at pH 9.0 in bacteriorhodopsin does not change its absorption spectrum but lowers the apparent p*K* of the alkaline transition to a blue-shifted chromophore from about pH 12.0 to 10.6. This effect is reversed by reducing the nitrotyrosine-26 to aminotyrosine which demonstrates that the protonation state of tyrosine-26 and the alkaline chromophore transition are correlated. Nitration of tyrosine-64 resulted in a shift of the purple complex from 570 to 535 nm at neutral pH. The alkaline transition p*K* of such a nitrated membrane was below 10 but was clearly independent of the protonation state of tyrosine-64 because it is not reversed by reduction of the nitrotyrosine. Nitrotyrosine-26 showed spectral properties similar to L-nitrotyrosine in aqueous environment while nitrotyrosine-64 showed only a 360-nm absorbance in the apomembrane but not in the retinal-containing membrane. Both tyrosines are accessible to water-soluble reagents.

Bacteriorhodopsin (bR),[1] the only protein in the purple membrane of *Halobacterium halobium*, functions as a light-driven proton pump [for a review, see Stoeckenius et al. (1979) and Stoeckenius & Bogomolni (1982)]. The bR molecules are arranged within the membrane in a rigid hexagonal lattice, and structural analysis by electron microscopy suggests a three-dimensional structure with seven helices spanning the membrane (Unwin & Henderson, 1975). The primary sequence is known (Ovchinnikov et al., 1979; Khorana

† From the Cardiovascular Research Institute and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143. *Received December 1, 1983.* This work was supported by NIH Program Project Grant GM-27057 and NASA Grant NSG-7151.

[1] Abbreviations: bR, bacteriorhodopsin; bO, bacterioopsin; pm, purple membrane; TNM, tetranitromethane; N-Tyr, nitrotyrosine; bR-TNM-d, bR modified with TNM in the dark; bR-TNM-l, bR modified with TNM in the light; HPLC, high-pressure liquid chromatography; CT, chymotrypsin; CNBr, cyanogen bromide; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate.

0006-2960/84/0423-6195$01.50/0