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Expression and Initial Characterization of Five Site-Directed Mutants of the N-Terminal Half-Molecule of Human Transferrin†

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ABSTRACT: Five site-directed mutants of the N-terminal half-molecule of human serum transferrin have been expressed in baby hamster kidney cells and purified to homogeneity. Expression levels and overall yields varied considerably from the wild-type protein, depending on the mutant in question. The mutants are D63S, D63C, G65R, K206Q, and H207E and are based on mutations observed in a variety of transferrins of known sequence. Their molecular masses, determined by electrospray mass spectrometry, agree with theory, except for the D63C mutant, which appears to be cysteinylated. All mutants bind iron but with varying affinities; qualitatively, in increasing order D63S ≈ D63C ≈ G65R ≪ wild type ≤ H207E ≪K206Q. In general, reduction of formal negative charge within the binding cleft shifts the visible spectral maximum of the iron complex toward the blue and reduces the affinity for iron, and increasing the formal negative charge shifts the visible maximum toward the red and increases the affinity for iron. The K206Q mutant is exceptional inasmuch as its visible maximum shows a blue shift, but its affinity for iron is the greatest of all of the mutants studied. All mutants reported, in addition to the wild-type protein, exhibit very similar visible molar extinction coefficients for the iron complex and very similar changes in extinction coefficients at 240 nm on binding Fe(III) or Ga(III). These results suggest that in all cases the bound metal ion is coordinated by two tyrosyl side chains.

The transferrins (siderophilins) comprise a group of ca. 80-kDa glycoproteins found in the blood plasma and body fluids of vertebrates (Harris & Aisen, 1989; Chasteen & Woodworth, 1990) and recently found in the larvae of an insect (Bartfeld & Law, 1990). To date, the X-ray crystal structures have been reported for lactoferrin from human milk (Anderson et al., 1989, 1990) and transferrin from rabbit blood serum (Bailey et al., 1988). These structures reveal that both proteins consist of two lobes of approximately equal size connected by a short peptide. Each lobe consists of two domains defining a deep cleft in which resides a binding site for iron or many other tri- and divalent metal ions. The cleft also binds a synergistic anion, usually carbonate, but it can adapt to many other small carboxylic acids containing a second electron-donor group (Schlabach & Bates, 1975; Woodworth et al., 1975; Dubach et al., 1991). The crystallographic studies reveal that the ligands to the bound Fe(III) or bound Cu(II) (Smith et al., 1991) are the phenolate oxygens of two tyrosyl side chains, a ring nitrogen of the imidazole side chain of a histidine, the carboxylate side chain of an aspartyl residue, and two oxygens from the bound carbonate. Previously reported waters of hydration (Koenig & Schillinger, 1969; Villafranca et al., 1976) appear to be absent from the bound metal ions.

The physiological functions of the plasma transferrins appear to be to shuttle iron in a soluble nontoxic form among the organs of the body, e.g., intestine, liver, bone marrow, and reticuloendothelial system, and to serve a general immunological role by depriving microorganisms of essential iron (Bullen et al., 1990). Physical–chemical studies of these proteins include UV-vis, epr, NMR, CD, and Raman spectroscopies of their complexes with various metal ions and synergistic anions (Harris & Aisen, 1989) and microcalorimetric and reaction kinetic measurements of complex for-

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mation and breakdown (Cowart et al., 1986; Bates & Schlabach, 1973; Kojima & Bates, 1981; Bali & Harris, 1990; Brandts & Lin, 1990).

Recently the expression and preliminary characterization of a recombinant form of the N-terminal half-molecule (N-terminal lobe, hTF/2N)[1] have been described (Funk et al., 1990). In this paper we describe the expression and initial characterization of five site-directed mutants of hTF/2N based on sequence comparisons with other known transferrin sequences.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium–Ham F-12 nutrient mixture (DMEM–F-12) was obtained with and without phenol red from Sigma. Defined and supplemented newborn calf serum (cat. no. A-2151-D) was obtained from Hyclone and pretested to assure adequate growth of BHK cells. The serum replacement Ultraser G came from either Gibco or Serva. A penicillin/streptomycin sulfate solution was from Gibco. Low-viscosity sodium alginate was purchased as a sterile solution from Bellco Glass. Cellift antifoam reagent was from Ventrex lab. Corning expanded surface roller bottles and Dynatech Removawells were obtained from a local distributor. All of the chromatographic resins, DEAE-Sephacel, Sephacryl S-100 HR, and Polyanion SI were from Pharmacia. Methotrexate from Cetus was purchased at a local hospital pharmacy. Immersible CX Ultrafilters were obtained from Millipore. Centricon 10 microconcentrators and PM-10 ultrafiltration membranes were from Amicon. Rabbit anti-mouse immunoglobulin G was purchased from Organon Teknika. All chemicals and reagents were analytical grade or purer.

A monoclonal antibody designated $\alpha HT+N_1$ was prepared in our laboratory and found to be specific for the amino-terminal lobe of transferrin. A complete description of this antibody is given elsewhere (Mason & Woodworth, 1991). This antibody has no reactivity with bovine transferrin.

Production of Amino Acid Substitution Mutants of hTF/2N. The production of hTF/2N mutants was accomplished via two techniques. The D63S substitution was carried out by the method of Nelson and Long (1989). A *Hpa*II/*Bam*HI fragment from the 5′ end of the hTF/2N coding sequence was subcloned into pUC18 and used as a template for a two-step, PCR-based mutagenesis procedure. The resulting DNA fragment was recloned into M13mp18 and the sequence of the mutant construction was confirmed by dideoxy sequence analysis. The fragment was released from the double-stranded form of the sequencing vector by digestion with *Xba*I and *Bam*HI and then ligated to a *Bam*HI/*Hin*dIII fragment from the original hTF/2N construction to produce a full-length D63S-hTF/2N coding sequence. The fidelity of this splicing was confirmed by restriction digestion analysis, and the DNA coding for this mutant was cloned into pNUT as previously described (Funk et al., 1990).

The substitution mutants D63C, G65R, K206Q, and H207E were produced by subcloning the entire hTF/2N coding sequence into M13mp18, which was then used as a template for oligonucleotide-directed mutagenesis (Zoller & Smith, 1983) using the *dut*⁻, *ung*⁻ selection procedure (Kunkel, 1985). After mutagenesis, the entire coding sequence for the mutant constructions was confirmed by dideoxy sequence analysis using sequencing primers targeted along the length of the coding sequence at 250-bp intervals. The desired coding sequences were then released by restriction digestion, made blunt, and ligated into pNUT as before (Funk et al., 1990).

Cells and Cell Maintenance. Baby hamster kidney (BHK) cells were transfected with the expression vector pNUT-hTF/2N, which encodes the natural signal sequence and the amino-terminal half-molecule lobe (hTF/2N) of human serum transferrin followed by two stop codons. This expression system has been thoroughly described (Funk et al., 1990). Frozen stocks of BHK cells transformed by the pNUT-hTF/2N plasmid were stored in liquid nitrogen in 95% fetal calf serum and 5% dimethyl sulfoxide (DMSO). Cells ($\sim 1 \times 10^6$) were initially brought up in DMEM–F-12 containing 5% newborn calf serum, penicillin (100 units/mL), and streptomycin (100 μg/mL). Methotrexate (500 μM) was used routinely in all cultures in which cell stocks were being stored at −190 °C. Methotrexate was absent from media for cells being expanded for mass culture. In a typical experiment, cells were passed at ~80% confluence using Versene. Sequential passage of the original cells was to five 100-mm dishes (8-mL volume), then to five T-175 flasks (30-mL volume), and finally to five expanded surface roller bottles (200-mL volume). At the T-175 passage a serum substitute, Ultraser G, at a level of 1% was used instead of 5% fetal calf serum in DMEM–F-12 lacking phenol red. Cells in the roller bottles were kept in a Bellco cell production roller apparatus set at a speed of 1 rpm.

Alginate bead entrapment, isolation of the recombinant hTF/2N, and the radioimmunoassay to determine the levels of hTF/2N have been described in detail (Funk et al., 1990; Mason et al., 1991). $NaDodSO_4$–PAGE and urea–PAGE were as previously described (Funk et al., 1990; Brown-Mason & Woodworth, 1984).

Molecular Weights. The molecular weights of the various hTF/2Ns were determined by electrospray mass spectroscopy at the Biological Analysis Research Unit, Department of Biological Sciences, University of Salford, Salford M5 4WT, England. The protein samples had been exchanged into 50 mM NH_4HCO_3 and then lyophilized several times, the residue having been redissolved in H_2O after each lyophilization.

Spectra. Visible spectra were recorded on a Cary 219 spectrophotometer with the apoprotein serving as the reference for the iron-containing protein. This approach served to correct for the increasing light scattering by the protein samples toward the blue end of the spectrum and to subtract out the Soret band from slight amounts of contaminating heme proteins in some of the samples. The ϵ_{max} values were calculated from the visible spectra or from the initial slope of titrations of the apo form with $Fe(NTA)_2$. Ultraviolet spectra were recorded on the same instrument with an H_2O reference. ΔA_{240} values were calculated by comparison of spectra of the apo and Fe forms of the protein or from the initial slope of difference spectral titrations of the apo form with Ga(III) (Harris & Pecoraro, 1983) and are initial estimates. Initial slopes were used in order to assure complete binding of the Ga(III) to the protein.

Results and Discussion

Since the original report, we have substantially increased the production levels of recombinant hTF/2N from baby hamster kidney cells transfected with the vector pNUT-hTF/2N (Funk et al., 1990; Mason et al., 1991). Figure 1 gives comparisons for the production rates and accumulation of various mutant forms of hTF/2N. Purified mutant proteins amounted to 20 mg of D63C, 104 mg of D63S, 96 mg of G65R, 81 mg of K206Q, and 51 mg of H207E. Maximum production levels ranged from 7 μg/mL of medium for the

[1] Abbreviations: hTF/2N, recombinant N-terminal half-molecule of human transferrin, ending at Asp 337; NTA, nitrilotriacetate; BHK, baby hamster kidney cells.

Table I: Comparison of Conserved Sequences of Five Transferrins[f]

	# 65	100	#125	193	209	255
Lobe	*	*	?# +###	*	??	*
hTF-N[a]	VTLDAG	YYAVAV	SCHTGLGRSAGWNIPI	YSGAFK	KHST	HTVVAR
LTF-N[b]	VTLDGG	YYAVAV	SCHTGLGRTAGWNVPI	YSGAFK	RHST	HAVVAR
MTF-N[c]	ITLDGG	YYAVAV	SCHTGINRTVGWNVPV	YSGAFR	KHST	HAVVVR
pTF-N[d]	VTLDAG	YYAVAV	SCHTGLGRSAGWIIPM	YSGAFN	KHST	HAVVAR
OTF-N[e]	ISLDGG	YYAVAV	SCHTGLGRSAGWNIPI	YSGAFH	KHTT	HAVVAR
	392	431	457	522	537	590
hTF-C[a]	MSLDGG	YFAVAV	SCHTAVGRTAGWNIPM	YTGAFR	KHQT	HAVVTR
LTF-C[b]	MSLDGG	YLAVAV	SCHTAVDRTAGWNIPM	YTGAFR	KDVT	HAVVSR
MTF-C[c]	VTLSGE	YYVVAV	SCHAGFGSPAGWDVPV	YRGAFR	RHTT	HAVMVR
pTF-C[d]	MSLDGG	YLAVAV	SCHTAVDRTAGWNIPM	YTGAFR	KDQV	HAVVAR
OTF-C[e]	VALDGG	YFAVAV	SCHTAVGRTAGWVIPM	YTGALR	QHST	HAVVVR

[a] N- and C-terminal lobes of human transferrin (MacGillivray et al., 1983; MacGillivray et al., 1982). [b] N- and C-terminal lobes of human lactoferrin (Metz-Boutigue et al., 1984). [c] N- and C-terminal lobes of human melanoferrin (Rose et al., 1986). [d] N- and C-terminal lobes of pig transferrin (Baldwin & Weinstock, 1988). [e] N- and C-terminal lobes of chicken ovotransferrin (Jeltsch & Chambon, 1982; Williams et al., 1982). [f] Legend: (*, bold) ligands to iron; (+, bold) ligand to anion; (#, bold) H-bonding in the active site; (?, bold) other side chains possibly involved in metal and anion binding. Sequence positions have been normalized to human serum transferrin.

D63C mutant to 34 μg/mL of medium for the G65R mutant. At this juncture the basis for different levels of expression, e.g., differing efficiencies of transfection or cellular export, is not known. Purification of the recombinant proteins has been enhanced by substitution of Ultraser G for bovine calf serum in the incubation medium, immediate concentration of harvested medium by vacuum ultrafiltration with Millipore immersible CX units, step elution of bound proteins from DEAE-Sephacel, rapid gel filtration on Sephacryl S-100 HR, and final removal of trace contaminants by FPLC on Polyanion SI when required. Final recoveries of purified proteins ranged between 20 and 50% of the protein in the media. This approach has led to total elimination of the "minor form" originally observed at the FPLC stage (Funk et al., 1990). The differences in levels of expression and yields of purified proteins are important for planning the scale of mass cultures required to yield a given amount of protein.

Site-directed mutants were based primarily on amino acid substitutions found on comparison of highly conserved sequences in five different transferrins. These regions are compared in Table I. Amino acid sequence numbers are based on human serum transferrin as the reference protein. The D63S mutant was based on the naturally occurring mutation found in the C-terminal lobe of melanotransferrin (Rose et al., 1986), which has been speculated to abrogate normal iron binding (Chasteen & Woodworth, 1990). The D63C mutant was a logical extension of the D63S mutant with the added goal of possibly modifying metal ion selectivity. The G65R mutant mimics that found in the C-terminal lobe of the serum transferrin of a patient in the United Kingdom (Evans et al., 1982). The K206Q mutation occurs naturally in the C-terminal lobe of ovotransferrin from hen egg white (Jeltsch & Chambon, 1982; Williams et al., 1982). The H207E mutation is based on this substitution in the N-terminal lobe of human lactoferrin (Metz-Boutigue et al., 1984), although the accompanying K206R substitution in the latter protein was not incorporated into the mutant hTF/2N.

All hTF/2N isolates are iron saturated. Iron is rapidly removed from the proteins with 0.5 M acetate, 1 mM EDTA, and 1 mM NTA, pH 5. Only the K206Q mutant required more stringent conditions, either a longer period, e.g., 16–24 h, or adjustment of the above buffer to pH 4 and the addition

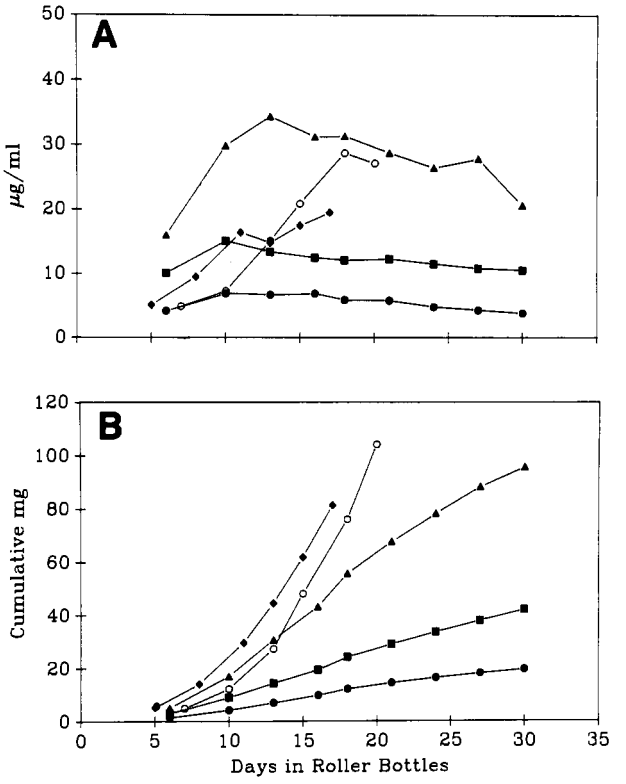

FIGURE 1: Production rate expressed as micrograms per milliliter (panel A) and total cumulative milligrams collected (panel B) of the various mutant hTF/2Ns produced by BHK cells in continuous production. The cells expressing mutants D63C (●), G65R (▲), and H207E (■) were entrapped in alginate beads in two expanded surface roller bottles. On the days indicated ~400 mL of medium was collected and assayed for recombinant protein. The cells expressing mutants K206Q (◆) and D63S (○) were cultured in five expanded surface roller bottles. On the days indicated ~1000 mL of medium was harvested and assayed for recombinant protein.

Table II: Spectral and Iron-Binding Characteristics of Recombinant Wild Type (WT) and Mutants of hTF/2N

protein	λ_{max} (nm)	λ_{min} (nm)	A_{max}/A_{min}	A_{278}/A_{max}	ϵ_{max} (mM)	$\Delta\epsilon_{240}$ (mM)	Fe binding rel to WT
WT	473	404	1.38	22	2.5	19	
D63S	420	368	1.33	21	2.5	19	$\ll$
D63C	440	388	1.28	23	2.6	19	$\ll$
G65R	468	410	1.31	29	1.9	15	$<$
K206Q	460	400	1.42	24	1.9	21	$\gg$
H207E	484	412	1.46	28	2.4	23	$\geq$

Table III: Mass Analysis of Recombinant Mutants of hTF/2N

protein	theoretical mass	experimental mass
wild type	37 151	37 151
D63S	37 123	37 127
D63C	37 139	37 259[a]
G65R	37 250	37 249
K206Q	37 154	37 151
H207E	37 143	37 142

[a] Mass is high by 120 amu, consistent with cysteinylation of C63.

of 1 mM deferoxamine. The apoproteins, thoroughly exchanged into 0.1 N KCl, were titrated with Fe(III)$(NTA)_2$. Each one recovered the original color and intensity of the iron complex. Some samples were titrated with Ga(III) and the binding was followed by UV difference spectroscopy. These titrations are curvilinear, so initial slopes were used to estimate $\Delta\epsilon_{240}$ values. The data are being evaluated further to establish K_d's for gallium binding to the various mutants. Table II gives λ_{max}, λ_{min}, A_{max}/A_{min} and A_{278}/A_{max} ratios, ϵ_{max}, and estimates of $\Delta\epsilon_{240}$ for the various hTF/2Ns. The λ_{max} is inversely proportional to the energy of the charge-transfer band for the Fe(III)–hTF/2N–(CO_3) complex. The spectral ratios are indicative of the degree of iron saturation and are within normal limits (Funk et al., 1990). The ϵ_{max} and $\Delta\epsilon_{240}$ values suggest that bound Fe(III) or Ga(III) atoms are each bound by two tyrosyl residues in the active site of the protein, i.e., the magnitudes are close to those reported for model complexes and holotransferrin (Tan & Woodworth, 1969; Pecoraro et al., 1981).

Qualitative information regarding the binding affinities of the various forms of hTF/2N for iron in the Fe(III)–nTF/2N–(CO_3) ternary complex was deduced from the behavior of the proteins on urea–polyacrylamide gels. Figure 2 shows that the ternary complexes of the D63S, D63C, and G65R mutants all migrate identically to the corresponding apoproteins. The conclusion is that these mutants have relatively low affinities for iron and therefore cannot compete with the denaturing forces of the 6 M urea in the gel. On the other hand the wild type and the K206Q and H207E mutants each retain their bound iron on the urea–polyacrylamide gels, as evidenced by their migrating more rapidly than the corresponding apoproteins.

Accurate determination of molecular mass by electrospray mass spectroscopy established that the designed mutant forms of hTF/2N had indeed been expressed. Table III lists the expected and experimentally determined masses of these proteins. In all cases except the D63C mutant, experiment and theory agree within experimental error. The D63C mutant protein gives a mass 120 ± 2 amu higher than theory, consistent with the cysteinylation of C63. A number of minor peaks of higher mass appear in the mass spectra of all of the proteins. Most prominent are peaks at ΔM = +97.6 (± 1.4) amu and +266.9 (± 2.9) amu. The first is consistent with binding of sulfate or phosphate and the second with binding of dipotassium nitrilotriacetate, used for removal of bound iron. Retention of these anions is consistent with the known binding of synergistic anions to the metal-free transferrins (Harris, 1985). The H207E mutant contains a minor component

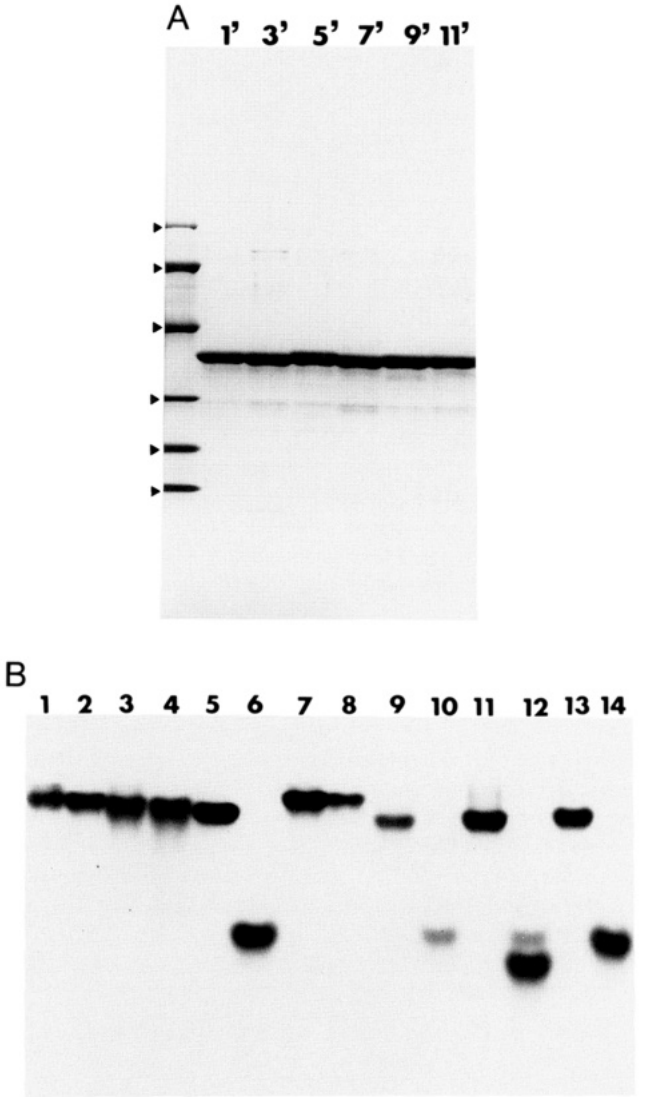

FIGURE 2: Electrophoresis of hTF/2N and the various mutants of hTF/2N on a $NaDodSo_4$–polyacrylamide gel (5–12% gradient of acrylamide) under reducing conditions (panel A) and on a urea–polyacrylamide gel (panel B). The bands in both cases are visualized with Coomassie blue. Samples on the $NaDodSO_4$–polyacrylamide gel are Bio-Rad low M_r standards with molecular weights (top to bottom) of 97 400, 66 200, 45 000, 31 000, 21 500, and 14 400; lane 1′, D63S mutant; lane 3′, D63C mutant; lane 5′, wild-type hTF/2N; lane 7′, G65R mutant; lane 9′, K206Q mutant; lane 11′, H207E mutant. Samples on the urea–polyacrylamide gel are lane 1, apo-D63S; lane 2, FeD63S; lane 3, apo D63C; lane 4, Fe D63C; lanes 5 and 13, apo hTF/2N; lanes 6 and 14, Fe hTF/2N; lane 7, apo G65R; lane 8, Fe G65R; lane 9, apo K206Q; lane 10, FeK206Q; lane 11, apo H207E; and lane 12, FeH207E. Approximately 10 μg of each sample was run.

(<15%) with a ΔM = −114.8 amu, consistent with loss of the C-terminal Asp residue. The K206Q mutant contains three minor components (<10%) with masses of 26 770, 26 937, and 30 000. However, no bands of these M_rs has seen on $NaDodSO_4$–PAGE. The origin of these species is not understood at this time.

Certain hypotheses can be formulated on the basis of the data accumulated to date on hTF/2N and its various mutants. All of the mutated residues lie in the binding-site cleft of the protein (Anderson et al., 1989; Bailey et al., 1988), D63 being a ligand to the bound metal and G65 lying two residues C-terminal to it. The residues K206 and H207 lie at the bottom of the interdomain cleft and are highly conserved in both lobes of many transferrins (see Table I). In human lactoferrin, which binds iron some 2 orders of magnitude more tightly than the serum protein (Harris, 1989), H207 becomes E207 (N-terminal lobe) and H535 becomes D535 (C-terminal lobe). In ovotransferrin K534 becomes Q534 (C-terminal lobe) and this site binds metals about 2 orders of magnitude more tightly than the site in the N-terminal lobe in which K206 is conserved (Ulundu, 1989). Mechanistically, one can view these changes in metal ion affinity as resulting from different degrees of charge repulsion by the 206/207 doublet. K206/H207 potentially bears a formal charge of 2+, Q206/H207 a charge of 1+, and R206/E207 or K534/D535 a charge of 0. Thus, the higher the positive charge in this region, the lower the binding affinity. In the case of D63, mutation to S63 *decreases* the formal negative charge by 1 unit. Cysteinylation of the D63C mutant results also in a net reduction of the formal negative charge by 1 unit.

From Table II certain generalizations can be made. Decreasing the negative charge in the binding-site region tends to shift the visible maximum toward the blue end of the spectrum and to weaken iron binding, whereas decreasing positive charge in this region tends to shift the visible maximum toward the red and to increase the affinity for iron. The exception is the K206Q mutant, which shows the highest affinity of any of the recombinant hTF/2Ns reported here but shows a blue shift in the visible spectrum. All reported forms show similar values for ϵ_{max} and $\Delta\epsilon_{240}$, which suggests that two tyrosyl side chains ligate each bound metal ion regardless of the amino acid substitutions. Obviously these considerations are incomplete, but the data show that conservative single amino acid substitutions in hTF/2N can profoundly affect the iron-binding properties of the protein. Whether these effects are fundamentally or thermodynamic or kinetic origin has yet to be established.

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Differences in the Binding Mechanism of RU486 and Progesterone to the Progesterone Receptor†

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ABSTRACT: The binding mechanism of the antagonist RU486 to the progesterone receptor was compared with that of the agonists progesterone and R5020. Both progesterone and RU486 bound to the receptor with a Hill coefficient of 1.2, indicating the binding of each ligand is positive cooperative. However, when each ligand was used to compete with [^{3}H]progesterone for binding to the receptor at receptor concentrations near 8 nM, at which the receptor is likely a dimer, the competition curve for RU486 was significantly steeper than the curves for progesterone and R5020 ($p < 0.001$). This indicated that a difference in the binding mechanism of RU486 and progesterone can be detected when both ligands are present. In contrast, at receptor concentrations near 1 nM, at which the receptor is likely a monomer, the competition curves for all three ligands were indistinguishable ($p = 0.915$). These results indicate that RU486 and agonists have different binding mechanisms for the receptor and further suggest that this difference may be related to site–site interactions within the receptor.

The antagonist RU486 binds to mammalian progesterone receptors and blocks the actions of progesterone in vivo (Philibert, 1984; Philibert et al., 1985; Moguilewsky & Philibert, 1985; Baulieu, 1989). RU486 also has some progestin activity when administered to castrate animals or postmenopausal women (Gravanis et al., 1985; Koering et al., 1986), which suggests it is an antagonist with partial agonist properties. Many characteristics of RU486-bound receptors have been studied to determine precisely how RU486 exerts its complex biological effects. RU486 binds with a similar or higher affinity to the receptor than does progesterone (Philibert, 1984; Moguilewsky & Philibert, 1985; Gravanis et al., 1985; Hurd & Moudgil, 1988). The RU486-bound progesterone receptor binds to its hormone response element with an affinity similar to that of the agonist-bound receptor (Bailly et al., 1986; El-Ashry et al., 1989). Like progesterone, RU486 promotes the nuclear association and oligomerization of receptors (Guiochon-Mantel et al., 1989). RU486 stabilizes the binding of hsp90 to the calf progesterone receptor (Moudgil & Hurd, 1989; Hurd et al., 1991) and increases the proportion of the 6S form of the progesterone receptor (Mullick & Katzenellenbogen, 1986). In the gel-shift assay, the RU486-bound receptor produces a band with a mobility different from that of the agonist-bound receptor (El-Ashry et al., 1989; Meyer et al., 1990). The agonist- and RU486-bound receptors also exhibit differential sensitivity to sulfhydryl group modification (Moudgil et al., 1989; Hurd et al., 1991). Finally, the RU486-bound progesterone receptor blocks transcription activation by the agonist-bound receptor (Guiochon-Mantel et al., 1988). It has been suggested that RU486 induces a different conformation in the progesterone receptor than do the agonists progesterone and R5020. However, most of the above investigations infer that the conformation of the RU486-bound receptor is different from the agonist-bound receptor by studying a property one or more steps removed from the interaction of the ligand with the protein. Furthermore, no characteristic of the binding mechanism between RU486 and the receptor has been found which correlates with the mixed antagonist/agonist activities of this compound.

In this report, the binding of RU486 to the calf uterine progesterone receptor has been examined with particular emphasis on how the presence of the antagonist affects the binding of the agonist progesterone. The results show that each ligand, when bound in the absence of the other, exhibits a similar binding mechanism. On the other hand, when both classes of ligand are present, it is apparent that RU486 interacts differently with the receptor than do the agonists progesterone and R5020. The results also show that the observed difference in the binding mechanism is dependent on the receptor concentration. Finally, the results provide evidence that site–site interactions play an important role in the conformational changes of the progesterone receptor.

Experimental Procedures

Materials

[1,2-^{3}H]Progesterone (40–60 Ci/mmol) was obtained from New England Nuclear (Boston, MA). [^{3}H]RU486 (46.8 Ci/mmol) and unlabeled RU486 were obtained from Roussel-Uclaf (Romainville, France). Other unlabeled steroids were obtained from Steraloids (Wilton, NH) or Sigma Chemical Co. (St. Louis, MO). Charcoal (Norit A) was obtained from Fisher (Livonia, MI). All other chemicals were reagent grade.

Methods

Preparation of Cytosol. Cytosol was obtained as described by Weichman and Notides (1977). Calf uteri obtained from

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