Williams, J., Elleman, T. C., Kingston, B., Wilkins, A. G., & Kuhn, K. A. (1982) *Eur. J. Biochem. 122*, 297–303.
Woodworth, R. C., Virkaitis, L. M., Woodbury, R. G., & Fava, R. A. (1975) in *Proteins of Iron Storage and Transport in Biochemistry and Medicine* (Crichton, R. R., Ed.) pp 39–50, North-Holland Publishing Co., Amsterdam.
Zoller, M. J., & Smith, M. (1983) *Methods Enzymol. 100*, 468–500.

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

D. F. Skafar
Department of Physiology, Wayne State University School of Medicine, 540 East Canfield, Detroit, Michigan 48201
Received August 26, 1991; Revised Manuscript Received September 23, 1991

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

† This research has been supported by NSF Grant DCB-8716044.

0006-2960/91/0430-10829$02.50/0

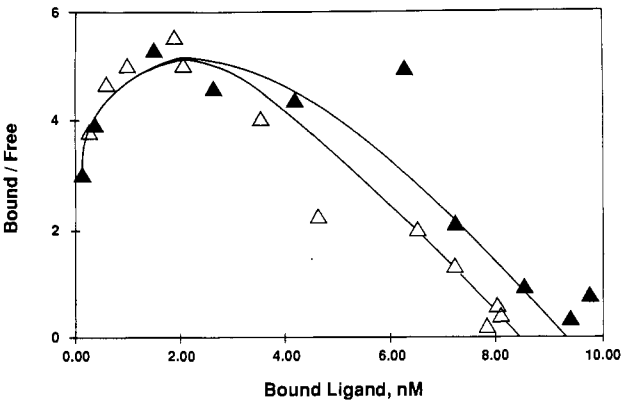

FIGURE 1: Scatchard analysis of the binding of [^{3}H]progesterone (△) and [^{3}H]RU486 (▲) to calf uterine cytosol. These data are typical of four independent experiments at receptor concentrations of 7.7 nM and greater. The corresponding Hill coefficients were obtained from the slopes of the Hill plots, where Y is the degree of saturation of the receptor and F is the concentration of free ligand. Values of 1.27 (△) and 1.18 (▲) were obtained.

a local slaughterhouse were trimmed, frozen, and stored at −70 °C. The frozen uteri were pulverized in a stainless steel mortar and pestle. The powder was homogenized in ice-cold 40 mM Tris, 1 mM dithiothreitol, and 0.1 mM EDTA, pH 7.4, containing 0.2 mM phenylmethanesulfonyl fluoride (TDE buffer). The homogenate was centrifuged at 27000g for 10 min. The supernatant was removed and centrifuged at 220000g for 45 min to obtain cytosol.

Saturation Binding Analysis. Saturation binding analysis was performed as described by Skafar (1991). Briefly, 200-μL aliquots of cytosol were incubated with increasing concentrations of [^{3}H]progesterone or [^{3}H]RU486 for 2 h (progesterone) or 4 h (RU486) on ice. Nonspecific binding was measured using a parallel set of incubations containing a 200-fold molar excess of unlabeled ligand. All incubations contained a 20-fold molar excess of cortisol. Free progesterone and bound progesterone were separated by incubation with 100 μL of 1% charcoal and 0.01% dextran in the TDE buffer for 10 min; free RU486 and bound RU486 were separated by incubation with 100 μL of 2% charcoal and 0.02% dextran in TDE buffer for 20 min. Tubes were centrifuged for 5 min at 4000 rpm in an HS-4 rotor. The radioactivity in 100-μL aliquots of the supernatant was measured in a scintillation counter with 3 mL of scintillation fluid. Each point is the average of duplicate incubations.

Competitive Binding Analysis. Aliquots (200 μL) of cytosol were incubated with 40 nM [^{3}H]progesterone, 4 nM cortisol, and the indicated concentration of competitor for 20–24 h on ice. Nonspecific binding was measured in parallel incubations containing 4 μM unlabeled progesterone. Bound [^{3}H]progesterone was measured by the dextran-coated charcoal assay as described for the saturation binding analysis.

Results

The calf progesterone receptor binds progesterone with a maximum Hill coefficient of 1.22 ± 0.02, which is indicative of a positive cooperative binding mechanism (Theofan & Notides, 1984; Skafar, 1991). The binding mechanism of RU486 was investigated by Scatchard analysis (Scatchard, 1949) and determination of the Hill coefficient (Hill, 1910). Both progesterone and RU486 exhibit curved Scatchard plots at receptor concentrations of approximately 8 nM (Figure 1). The Hill coefficients for the binding of progesterone and RU486 to the receptor were indistinguishable: 1.2 ± 0.02 vs 1.23 ± 0.02, respectively (Table I).

Table I: Hill Coefficients of Binding [^{3}H]RU486 and [^{3}H]Progesterone to the Calf Uterine Progresterone Receptor[a]

ligand	Hill coefficient
[^{3}H]progesterone	1.20 ± 0.02
[^{3}H]RU486	1.23 ± 0.02

[a] Hill coefficients were obtained from the slope of the Hill plot (Hill, 1910) using binding data obtained as described under Methods. The values shown are the mean ± SE of four independent experiments at receptor concentrations of 7.7 nM and greater.

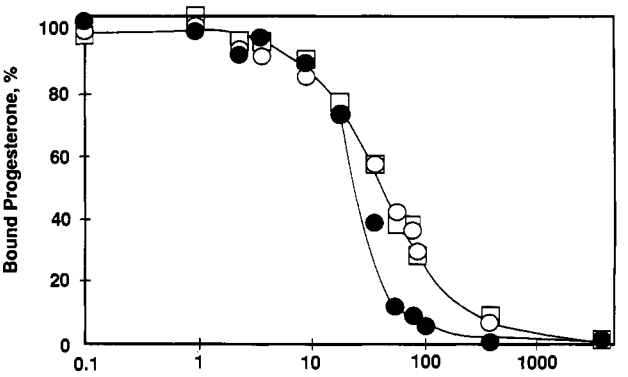

FIGURE 2: Competition of [^{3}H]progesterone binding to calf uterine cytosol by progesterone (○), R5020 (□), and RU486 (●) at a high receptor concentration. Specifically bound [^{3}H]progesterone was measured in the presence of the indicated concentration of competitor as described under Methods. Each point is the average of duplicate incubations; the experiment shown is typical of three independent experiments at receptor concentrations of 7.8–8.3 nM. The slopes of the linear portion of the curves of the pooled data were first compared using the change in the F ratio of the regression lines of the grouped vs ungrouped data and were found to be significantly different (p = 0.00434). The lines were also tested for parallelism using ANOVA and the differences between the antagonist and the agonists were found to be significant ($p < 0.001$).

The ability of one ligand to influence the binding of another was then examined by comparing the ability of increasing concentrations of unlabeled progesterone, R5020, and RU486 to compete with [^{3}H]progesterone for binding to the receptor at high and low receptor concentrations. At receptor concentrations near 8 nM, the agonists progesterone and R5020 exhibited nearly superimposable competition curves (Figure 2). The competition curve for RU486, however, was significantly different for receptor binding (p = 0.00434, Figure 2). The shift of the competition curve for RU486 to the left of those of the agonists indicated RU486 had a higher affinity for the receptor than the agonists. More importantly, the slope of the linear portion of the curve was significantly steeper ($p < 0.001$) than the slopes of the curves for progesterone and R5020. In contrast, at receptor concentrations between 0.6 and 1.7 nM, the competition curves for progesterone, RU486, and R5020 were indistinguishable (p = 0.915, Figure 3).

Discussion

Progesterone and RU486 bound to the receptor with Hill coefficients of 1.2 (Figure 1; Table I). Therefore, by these criteria each ligand, when analyzed independently, interacted in a positive cooperative manner with the progesterone receptor. This parallels the observations in vivo that RU486 has some progestin activity in the absence of progesterone (Gravanis et al., 1985; Koering et al., 1986). It also agrees with the results of previous studies on the binding of RU486 to progesterone receptors, which had not detected any difference in the binding mechanism of agonist and antagonist ligands (Philibert, 1984; Moguilewsky & Philibert, 1985; Hurd &

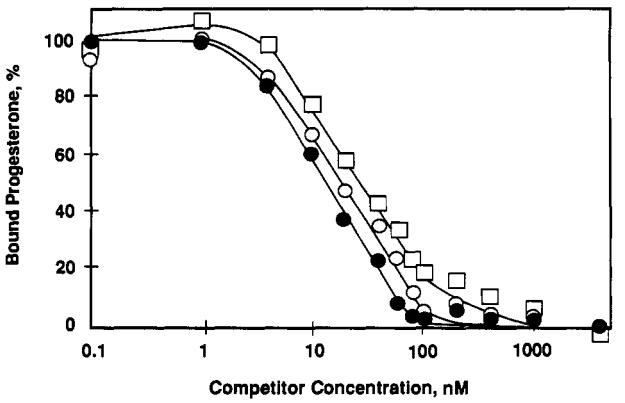

FIGURE 3: Competition of [^{3}H]progesterone binding to calf uterine cytosol by progesterone (○), R5020 (□), and RU486 (●) at a low receptor concentration. The assay was performed as described under Methods except that the cytosol was diluted with TDE buffer and 20 nM [^{3}H]progesterone was used. The experiment shown is typical of four independent experiments at receptor concentrations of 0.6–1.7 nM. The slopes of the linear portion of the displacement curves of the pooled data were compared as described in the legend to Figure 2 and were not found to be significantly different (p = 0.915).

Moudgil, 1988). However, in contrast with the present work, the binding mechanism of each class of ligand was reported to be noncooperative. In some cases, this may be due to the lower concentrations of receptor used in the earlier work, 3 nM (Philibert, 1984; Moguilewsky & Philibert, 1985), since the progesterone receptor exhibits its maximum Hill coefficient for [^{3}H]progesterone binding, 1.2, at receptor concentrations near 8 nM (Theofan & Notides, 1984; Skafar, 1991). The presence of molybdate in other experiments could potentially affect the Hill coefficient by stabilizing the 8S form of the receptor. Failure to observe cooperative binding has also been attributed to a lack of data points at low ligand concentrations (Hurd & Moudgil, 1988).

A Hill coefficient of 1.2 is indicative only of positive cooperativity; that is, the binding of one molecule of ligand helped the second molecule to bind. It does not necessarily indicate whether the conformational changes accompanying binding one ligand are identical to those accompanying binding of different ligands. To test that, binding studies in the presence of both ligands were performed. The slope of the competition curve for RU486 was different than the slopes for progesterone and R5020 at receptor concentrations near 8 nM (Figure 2). This indicated that the two classes of ligand interact differently with the receptor and strongly supports the idea that they produce different conformational changes in the receptor. The positive cooperativity of the calf progesterone receptor observed at this same receptor concentration, near 8 nM, is indicative of site–site interactions occurring within a dimer (Theofan & Notides, 1984; Skafar, 1991). The human progesterone receptor has been shown directly to dimerize in solution (DeMarzo et al., 1991). It was therefore possible that binding of the antagonist at one site could influence the binding of agonist at another. Indeed, the steeper slope of the competition curve for RU486 is consistent with a preference of the receptor to have RU486 occupy both sites of a dimer, rather than having progesterone and RU486 each occupy one of the two sites of a dimer. This is similar to the results of Meyer et al. (1990), who did not detect heterodimer formation between R5020-bound human progesterone receptor form B and RU486-bound human progesterone receptor form A in a gel-shift assay.

To test the idea that site–site interactions within a receptor dimer were important in producing the different slope of the competition curve for RU486, competition experiments were performed at low receptor concentrations. At receptor concentrations of about 4 nM, the Hill coefficient for [^{3}H]-progesterone binding is 1, indicating that site–site interactions are lost and the receptor is most likely a monomer (Skafar, 1991). At a receptor concentration of about 1 nM, no difference in the slope of the competition curves was detected (Figure 3). This supports the concept that site–site interactions are important in the binding mechanism of RU486 with the receptor. It may also explain in one case why the difference in the slope of the displacement curves had not been previously detected: in the earlier work, receptor concentrations of about 1.5 nM had been used (Gravanis et al., 1985). In another case, cytosol from T47D$_{CO}$ cells was used, which probably has a high concentration of receptor, and yet the displacement curves for R5020 and RU486 were superimposable (Horwitz, 1985). This may be due to the presence of molybdate, which as discussed above stabilizes the 8S form of the receptor and could therefore influence site–site interactions.

The interaction of RU486 with the progesterone receptor was strikingly similar to the binding of 4-hydroxytamoxifen to the calf estrogen receptor. Both estradiol and 4-hydroxytamoxifen bind to the estrogen receptor with Hill coefficients of 1.4–1.5. Furthermore, the slope of the displacement curve is steeper for 4-hydroxytamoxifen than for estradiol (Sasson & Notides, 1988). It would be interesting to determine whether the binding mechanism of 4-hydroxytamoxifen is dependent on the receptor concentration.

The difference in the binding mechanism of RU486 observed upon changing the receptor concentration may have important implications for its biological activity. Progesterone receptor levels vary among tissues and change in different physiological states, such as during the menstrual cycle (Bayard et al., 1978). It can be speculated that receptor concentration may be correlated with not only the sensitivity of an organ to a hormone, as has been observed for the chicken oviduct (Spelsberg et al., 1979a,b; Seaver et al., 1980), but also the biological activity of the hormone or ligand. In other words, at low receptor concentrations RU486 may have more agonist activity than at higher receptor concentrations.

The observed difference in the slope of the competition curves provides direct evidence for a difference between the interaction of RU486 and the agonists progesterone and R5020 with the progesterone receptor that produces different conformational changes in the receptor. Moreover, the difference in the binding mechanism observed when both classes of ligand were present and the similar mechanism observed when analyzed alone is consistent with the ability of RU486 to block progesterone action yet display some agonist activity in the absence of progesterone. Finally, the dependence of the slope of the competition curves on the concentration of receptor suggests that site–site interactions may play an important role in the conformation of the progesterone receptor and the biological activity of RU486.

ACKNOWLEDGMENTS

I thank Roussel-Uclaf for their generous gifts of RU486 and [^{3}H]RU486, Dr. Sam Brooks and Dr. Miriam Lifsics for their helpful comments on the manuscript, and Ms. Linda McCraw for her excellent typing.

REFERENCES

Bailly, A., Le Page, C., Rauch, M., & Milgrom, E. (1986) *EMBO J. 5*, 3235.

Baulieu, E. E. (1989) *Science 245*, 1351.

Bayard, F., Damilano, S., Robel, P., & Baulieu, E.-E. (1978) *J. Clin. Endocrinol. Metab. 46*, 635.

DeMarzo, A. M., Beck, C. A., Onate, S. A., & Edwards, D. P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 72.
El-Ashry, D., Onate, S. A., Nordeen, S. K., & Edwards, D. P. (1989) *Mol. Endocrinol. 3*, 1545.
Gravanis, A., Schaison, G., George, M., de Brux, J., Satyaswaroop, P. G., Baulieu, E. E., & Robel, P. (1985) *J. Clin. Endocrinol. Metab. 60*, 156.
Guiochon-Mantel, A., Loosfelt, H., Ragot, T., Bailly, A., Atger, M., Misrahi, M., Perricaudet, M., & Milgrom, E. (1988) *Nature 336*, 695.
Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M., & Milgrom, E. (1989) *Cell 57*, 1147.
Hill, A. V. (1910) *J. Physiol. (London) 40*, iv.
Horwitz, K. B. (1985) *Endocrinology 116*, 2236.
Hurd, C., & Moudgil, V. K. (1988) *Biochemistry 27*, 3618.
Hurd, C., Nakao, M., Eliezer, N., & Moudgil, V. K. (1991) *Mol. Cell. Biochem. 105*, 73.
Koering, M. J., Healy, D. L., & Hodgen, G. D. (1986) *Fertil. Steril. 45*, 280.
Meyer, M.-E., Pornon, A., Ji, J., Bocquel, M.-T., Chambon, P., & Gronemeyer, H. (1990) *EMBO J. 9*, 3923.
Moguilewsky, M., & Philibert, D. (1985) in *The Antiprogestin Steroid RU486 and Human Fertility Control* (Baulieu, E. E., & Segal, S. J., Eds.) p 87, Plenum, New York.
Moudgil, V. K., & Hurd, C. (1987) *Biochemistry 26*, 4993.
Moudgil, V. K., Anter, M. J., & Hurd, C. (1989) *J. Biol. Chem. 264*, 2203.
Mullick, A., & Katzenellenbogen, B. S. (1986) *Biochem. Biophys. Res. Commun. 135*, 90.
Philibert, D. (1984) in *Adrenal Steroid Antagonism* (Agarwal, M. K., Ed.) p 77, deGruyter and Co., Berlin.
Philibert, D., Moguilewsky, M., Mary, I., Lecaque, D., Tournemine, C., Secchi, J., & Deraedt, R. (1985) in *The Antiprogestin Steroid RU486 and Human Fertility Control* (Baulieu, E. E., & Segal, S. J., Eds.) p 49, Plenum, New York.
Sasson, S., & Notides, A. C. (1988) *Mol. Endocrinol. 2*, 307.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660.
Seaver, S. S., Van Eys, D. C., Hoffman, J. F., & Coulson, P. B. (1980) *Biochemistry 19*, 1410.
Skafar, D. F. (1991) *Biochemistry 30*, 6148.
Spelsberg, T. C., Boyd, P. A., & Halberg F. (1979a) *Adv. Exp. Med. Biol. 117*, 225.
Spelsberg, T. C., Thrall, C., Martin-Dani, G., Webster, R. A., & Boyd, P. A. (1979b) in *Ontogeny of Receptor and Reproductive Hormone Action* (Hamilton, T. H., & Clark, J. H., Eds.) p 31, Raven Press, New York.
Theofan, G. S., & Notides, A. C. (1984) *Endocrinology 114*, 1173.
Weichman, B., & Notides, A. C. (1977) *J. Biol. Chem. 252*, 8856.

Selecting High-Affinity Binding Proteins by Monovalent Phage Display†

Henry B. Lowman, Steven H. Bass, Nancy Simpson, and James A. Wells*

Department of Protein Engineering, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, California 94080

Received August 15, 1991; Revised Manuscript Received September 23, 1991

ABSTRACT: Variants of human growth hormone (hGH) with increased affinity and specificity for the hGH receptor were isolated using an improved phage display system. Nearly one million random mutants of hGH were generated at 12 sites previously shown to modulate binding to the hGH receptor or human prolactin (hPRL) receptor. The mutant hormones were displayed in a monovalent fashion from filamentous phage particles as fusions to the gene III product of M13 packaged within each particle. After three to six cycles of enrichment for hGH-phage particles that bound to hGH receptor beads, we isolated hGH mutants that exhibited consensus binding sequences for the hGH receptor. Residues previously identified as important for hGH receptor binding by alanine-scanning mutagenesis were more highly conserved by this selection method. However, other residues nearby were not optimal, and by mutating them, hormone variants having greater affinity and selectivity for the hGH receptor were isolated. This approach should be useful for those who wish to modify and understand the energetics of protein–ligand interfaces.

The design of variant hormones with enhanced receptor affinity and selectivity is important for dissecting the biological activities of hormones as well as creating new ones with greater therapeutic potential. Human growth hormone exhibits a variety of biological effects [for review see Isaksson et al. (1985)] that are initiated by binding to specific receptors, such as the hGH[1] receptor and hPRL receptor [for review see Hughes and Friesen (1987)]. Alanine-scanning mutational analysis (Cunningham & Wells, 1989, 1991) has identified sets of nonidentical but overlapping residues (epitopes) that modulate the binding of hGH to the extracellular domains of the hGH and hPRL receptor (called hGHbp and hPRLbp) (Figure 1). In one case, an alanine replacement at Glu174

† H.B.L. is supported by NIH Postdoctoral Grant GM13560-02.
* To whom correspondence should be addressed.

[1] Abbreviations: hGH, human growth hormone; hPRL, human prolactin; hGHbp, hGH binding protein consisting of the extracellular domain of the hGH receptor; hPRLbp, hPRL binding protein consisting of residues 1–211 of the hPRL receptor; Mab, monoclonal antibody; BSA, bovine serum albumin. Single mutants are designated by the wild-type residue (in single-letter code) followed by its position and the mutant residue. Multiple mutants are indicated by a series of single mutants separated by slashes; for example, E174S/F176Y denotes a double mutant in which Glu174 and Phe176 are converted to Ser and Tyr, respectively.

0006-2960/91/0430-10832$02.50/0