Thermodynamics of Follitropin Binding to Solubilized Calf Testis Receptor†

Barbara B. Sanborn, Thomas T. Andersen,* and Leo E. Reichert, Jr.
Department of Biochemistry, Albany Medical College, Albany, New York 12208
Received April 17, 1987; Revised Manuscript Received August 7, 1987

ABSTRACT: Thermodynamic parameters of follitropin binding to solubilized testicular receptors were measured in order to assess the forces involved in the binding reaction. Reversibility of follitropin binding to solubilized receptor decreased only 20% over the temperature range 4–24 °C, whereas earlier studies indicated reversibility of binding to membrane-bound receptor decreased by more than 40% over the same range [Andersen, T. T., Curatolo, L. M., & Reichert, L. E., Jr. (1983) *Mol. Cell. Endocrinol. 33*, 37–52]. Thermodynamic analysis of follitropin binding to solubilized receptors showed that the hydrophobic effect was important in the binding reaction. The mean values, at 25 °C, for ΔH and ΔS were −31.8 kcal/mol and −66.0 cal mol^{-1} K^{-1}, respectively, and ΔC_p was −3.0 kcal mol^{-1} K^{-1}. This is an unusually large heat capacity for protein–protein association reactions, indicating an enhanced role for the hydrophobic effect with the solubilized (compared to membrane-bound) receptor. Since glycerol was necessary to stabilize the solubilized receptor, we determined whether glycerol affected the thermodynamic parameters measured for the binding reaction. Control experiments, performed with membrane-bound receptor in the presence or absence of glycerol, indicated that ΔC_p actually decreased upon addition of glycerol (−0.8 kcal mol^{-1} K^{-1} in the presence of glycerol compared to −2.3 kcal mol^{-1} K^{-1} in the absence of glycerol). Thus, the large negative ΔC_p observed for the soluble receptor was a result of its removal from the membrane and was not due to the presence of glycerol. Results of this study establish that the hydrophobic effect is the most important force participating in the interaction of follitropin with its receptor, whether solubilized or in the membrane.

The pituitary glycoprotein hormone follitropin (FSH)[1] is composed of two polypeptide chains, each of which is glycosylated, each of which has many intramolecular disulfides, and each of which has been sequenced (Pierce & Parsons, 1981). FSH stimulates important testicular functions, such as spermatogenesis, after it binds to receptors on Sertoli cell membranes. A thermodynamic analysis of the membrane-bound receptor binding interaction with FSH was previously reported (Andersen et al., 1983) supporting a role for the hydrophobic effect, for which the driving force is the increased entropy of water released from the surface of hormone and receptor allowing the juxtaposition of nonpolar residues. Complications were noted when utilizing FSH and the membrane-bound form of the receptor for such studies, including a decrease in reversibility of hormone binding as a function of temperature. Our results, however, suggested that for FSH binding, the hydrophobic effect might be the first part of a two-step reaction and that a later step might involve essentially irreversible binding.

The FSH receptor in testis has been studied in forms other than when it is associated with the particulate form of the membrane. It has been solubilized with Triton X-100 (Abou-Issa & Reichert, 1977; Dias et al., 1981) and has been studied in a vesicular preparation (Branca et al., 1985) and as a buffer-soluble component of a cytosolic fraction (Dias & Reichert, 1982). Since nonreceptor components of the membrane (either lipid or protein) might lead to nonspecific or irreversible interactions with hormone, we hypothesized that FSH binding to detergent-solubilized receptors would be more reversible and less complex than that with membrane-bound receptor. This proved to be the case, and we report here the results of a thermodynamic analysis of follitropin binding to Triton X-100 solubilized receptors.

Materials and Methods

Frozen immature beef testes were obtained from local abattoirs. *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) was from Calbiochem-Behring (San Diego, CA), ovalbumin and bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO), poly(ethylene glycol) 6000 (PEG) was from Sigma Chemical Co. or Fisher Scientific (Springfield, NJ), and other chemicals were from Fisher Scientific. Na^{125}I was from Amersham Corp. (Arlington Heights, IL) or from New England Nuclear Research Products (Boston, MA).

Human follitropin (LER-1781-2, biological potency about 4000 IU/mg) was radioiodinated with Na^{125}I using lactoperoxidase, with hydrogen peroxide added at 2-min intervals in order to obtain approximately 60% incorporation as determined by the method of Greenwood et al. (1963). Radiolabeled follitropin of high bindability (approximately 35%, estimated with membrane-bound receptor; Branca et al., 1985) was obtained by polyacrylamide gel electrophoresis of the iodinated product (Schneyer et al., 1985). This procedure separated radiolabeled follitropin from follitropin subunits and high molecular weight contaminants. Membrane-bound receptor was prepared at 4 °C after decapsulation of thawed immature bovine testes and removal of the tunica albuginea. All isolation and purification steps were carried out at 4 °C. The parenchyma were twice ground in chilled homogenization buffer (0.2 g of parenchyma/mL of buffer) which contained 0.01 M Tris, 15% (w/v) sucrose, 0.001 M $CaCl_2$, 0.001 M $MgCl_2$, 0.001% (10 μM) 2-mercaptoethanol, 0.01% sodium azide, and 0.01 mM *p*-(hydroxymercuri)benzoate, using a Brinkmann Polytron homogenizer. The first homogenization used a 35-mm probe in 1000 mL for 30 s at full speed. The

† This study was supported by grants from the National Institutes of Health (HD16716 and HD13938).

[1] Abbreviations: FSH, follicle stimulating hormone; PEG, poly(ethylene glycol); TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/87/0426-8196$01.50/0

second used a 10-mm probe in 500 mL with the same time and speed. The homogenate was strained at least twice through cheesecloth before centrifugation at 7000*g* for 15 min, and the supernatant solution was strained through cheesecloth again before concentration in order to improve flow through the filtration apparatus. An Amicon hollow fiber apparatus (DC10) containing HI-50 fibers was used to concentrate the receptor relative to substances with molecular weights less than 50000. The concentrated retentate was diluted with 1 volume of rinse buffer (0.01 M Tris, 0.001 M $CaCl_2$, 0.001 M $MgCl_2$, and 0.01% sodium azide, pH 7.4 at 4 °C), reducing the sucrose content from 15% to 7.5%. The retentate was then concentrated to $^1/_{20}$th of the original volume with the hollow fiber apparatus (Dattatreyamurty et al., 1986). The final product from this procedure was frozen at −70 °C until used for assay or for solubilization. Proteolytic activity was minimized by carrying out the entire procedure quickly at 4 °C using isoosmotic sucrose buffers. Further, the 50000 molecular weight filtration step removes most proteases following homogenization. In addition, at least one protease inhibitor has been shown to complicate FSH binding assays (Dias et al., 1983), and therefore, such inhibitors were not utilized.

Detergent-solubilized receptor was obtained from the hollow fiber retentate by using a procedure described by Dias et al. (1981). Retentate was thawed and diluted with 1.5 volumes of buffer (0.10 M sucrose, 0.02 M TES, 0.005 M $MgCl_2$, and 0.01% sodium azide, pH 7.4 at 20 °C) and then centrifuged at 25000*g* for 1 h, and the supernatant was discarded. Solubilization buffer [30% glycerol, 0.01 M TES, 0.001 M $CaCl_2$, 0.001 M $MgCl_2$, 2.0% (w/v) Triton X-100, and 0.01% sodium azide, pH 7.4 at 20 °C) was added to the precipitate (1.25 mL/g of pellet), and the pellet was triturated with a Pasteur pipet and stirred 1 h at 4 °C. The suspension was centrifuged at 250000*g* for 60 min, and the supernatant was stored at −70 °C until assay.

Competitive binding assays measured inhibition of radioiodinated follitropin (as ^{125}I-hFSH) binding to receptor by increasing concentrations of unlabeled FSH. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled FSH while total binding was determined in the absence of unlabeled hormone. The concentration of labeled hormone was 3 ng/tube, with approximately 300000 cpm added per tube, while concentrations of unlabeled human FSH (LER-2030-3A, biological potency about 890 IU/mg), prepared by serial dilution, were between 0.152 and 4000 ng/tube. Solubilized receptor was always added last, to a final volume of 1.0 mL. The final assay volume for membrane-bound receptor assays was 0.5 mL. Triton X-100 concentration was maintained between 0.15% and 0.25%, since specific binding was stable throughout that range (data not shown). Assay buffer for solubilized receptors contained 30% glycerol, 20 mM TES, 5 mM $MgCl_2$, and 0.1% (w/v) ovalbumin at pH 7.4. Membrane-bound receptors were assayed in 50 mM TES, 0.1 M sucrose, 5 mM $MgCl_2$, and 0.1% (w/v) ovalbumin at pH 7.4, except that 30% glycerol (v/v) was substituted for 0.1 M sucrose in assays which tested the effect of glycerol on membrane-bound receptor binding. Membrane-bound receptor binding assays contained 4.5 mg of receptor protein, while approximately 0.44 mg of detergent-solubilized receptor protein was used in solubilized receptor binding assays. Protein was determined by the amido-Schwarz dye binding method (Schaffner & Weissman, 1973).

Assays were incubated in water baths at temperatures ranging from 4.5 to 30 °C and were maintained at the set temperature ±0.1 °C. The incubation was terminated after the binding reaction had reached steady state, at 19.5 h, using 12.5% (w/v) PEG as described previously (Dias et al., 1981). Assays in which the receptor had not been solubilized were terminated by filtration on 25-mm hydrophilic Durapore filters on a 12-slot Millipore filtration apparatus. Filters were wetted in 0.01 M Tris, 0.001 M $CaCl_2$, 0.001 M $MgCl_2$, and 0.01% sodium azide, pH 7.4, before use, and filtration was accomplished within 10 s in most cases. The filters were then placed in polypropylene tubes for counting.

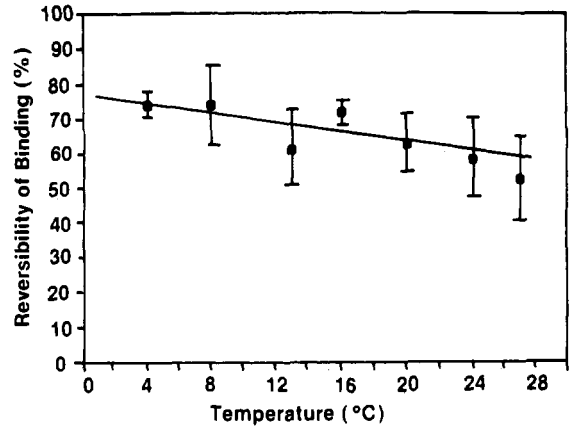

FIGURE 1: Reversibility of follitropin binding to solubilized receptors as a function of temperature. Reversibility represents the extent to which labeled follitropin can be displaced from detergent-solubilized receptors by the addition of unlabeled hormone 2 h after the beginning of incubation. Results plotted are the means ± SD of three assays, each of which used triplicate determinations.

Assessment of reversibility of hormone–receptor binding was performed as previously described (Andersen et al., 1983). Data from competitive binding assays were analyzed by using the LIGAND program of Munson and Rodbard (1980). Affinity constants generated by LIGAND were used for thermodynamic analysis of follitropin binding to receptors as previously described (Andersen et al., 1983).

RESULTS

The reversibility of FSH binding to detergent-solubilized receptor as a function of temperature is shown in Figure 1. Reversibility of binding decreased only slightly, from 75% (at 4 °C) to 55% (at 28 °C). The detergent-solubilized receptor preparation did not exhibit the extreme temperature-dependent irreversibility which had been observed previously (Andersen et al., 1983) with membrane-bound receptor.

After the enhanced reversibility of FSH binding had been established, thermodynamic studies were performed with the solubilized receptor. The inset to Figure 2 shows a curvilinear van't Hoff plot of the data obtained from competitive binding assays, indicating that the binding reaction was complex. Consequently, the data were plotted in terms of ΔG versus temperature and were fitted to a polynomial (Figure 2) as has been described previously with testis membranes (Andersen et al., 1983) and other systems (Ross & Subramanian, 1981; Waelbroeck et al., 1979). Equations for entropy and enthalpy were derived from this polynomial and are plotted as a function of temperature in Figure 3A. Values for heat capacity were also calculated and are shown in Figure 3B. All parameters related to ^{125}I-hFSH binding to solubilized receptor were highly dependent on temperature. The negative heat capacity (average of three determinations, −3.0 kcal mol^{-1} K^{-1}) was of greater magnitude than the value obtained with the membrane-bound form of the receptor (−1.9 kcal mol^{-1} K^{-1}; Andersen et al., 1983). These data are consistent with the interpretation that the hydrophobic effect is enhanced or more

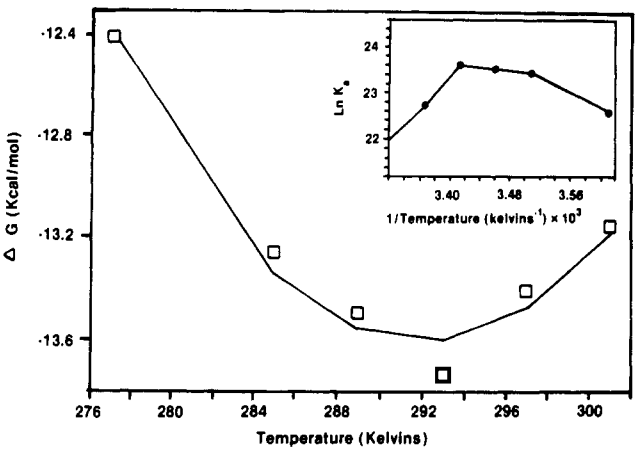

FIGURE 2: Free energy of follitropin binding to detergent-solubilized receptors. Since the van't Hoff plot (inset) was curvilinear, values of ΔG were calculated from the affinity constants and plotted as a function of temperature. A polynomial of the form $\Delta G = A + BT + CT^2$ was fitted to the data using the program POLFIT. The polynomial could then be differentiated to obtain equations for ΔH, ΔS, and ΔC_p. The polynomial was $\Delta G = 443 - 3.13T + 0.005T^2$. Squares depict data from binding assays, and the line represents the fitted polynomial.

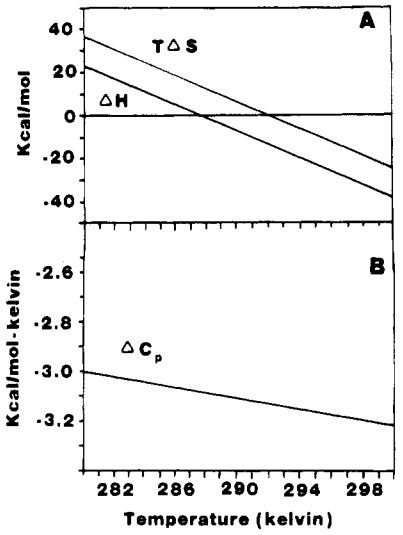

FIGURE 3: Thermodynamic parameters for detergent-solubilized follitropin receptor. The enthalpic (ΔH) and entropic ($T\Delta S$) contributions to the free energy are shown in panel A, while the change in heat capacity of the binding reaction is shown in panel B. The pronounced temperature dependence of ΔH is reflected in a large negative value for ΔC_p (−3.0 kcal mol^{-1} K^{-1} at 25 °C, average of three determinations of which the figure shown is one).

pronounced when using detergent-solubilized receptors.

Glycerol, which is important for stabilization of the solubilized receptor (Dias et al., 1981), reportedly leads to preferential hydration of proteins (Gekko & Timasheff, 1981b), and its effect on the thermodynamics of the FSH–receptor interaction was therefore examined. Since it was not possible to monitor the thermodynamics of binding to solubilized receptors in the absence of glycerol due to instability of the receptor prepared under these conditions of extraction, control experiments were devised in which glycerol was present or absent from membrane-bound receptors. Values of thermodynamic parameters as a function of temperature in the absence of glycerol are shown in Figure 4A,B and in the presence of glycerol in Figure 4C,D. ΔH and ΔS remain positive over higher temperatures in the presence of glycerol than in its absence, and the ΔC_p is less negative when glycerol is present. This confirms that the larger negative ΔC_p observed for the

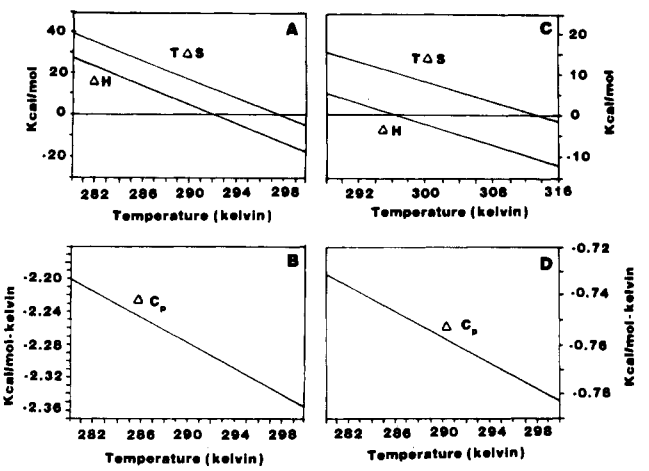

FIGURE 4: Thermodynamic parameters for follitropin binding to membrane-bound receptors in the presence or absence of glycerol. As in Figure 3, enthalpy and entropy contributions to ΔG are shown in panel A, and heat capacity is shown in panel B for the *absence* of glycerol. The value for ΔC_p at 25 °C is −2.3 kcal mol^{-1} K^{-1}. Enthalpy and entropy are shown in panel C and heat capacity in panel D for the *presence* of glycerol. The value for ΔC_p at 25 °C is −0.7 kcal mol^{-1} K^{-1}, a much lower value than when glycerol was absent.

Table I: Effects of Glycerol on FSH–Receptor Precipitation[a]

condition	final [glycerol] (v/v)	specific binding, mean ± SD (cpm)	assay volume (mL)
control	30	9415 ± 2992	1.0
less glycerol	15	9320 ± 1018	0.75
less glycerol	20	9457 ± 928	0.75
volume control	30	9701 ± 1206	0.75
dilution control	30	10053 ± 1230	0.75

[a] The results are the mean of three replicates, determined by subtraction of nonspecific binding (determined in the presence of excess unlabeled follitropin) from total binding (determined in the absence of unlabeled follitropin). All tubes contained 30% glycerol during 19.5 h of incubation. Incubation volume of the first set was 1.0 mL throughout, and glycerol concentration was 30% throughout, representing specific binding under normal conditions. Volume of the second, third, and fourth sets was 0.75 mL, and these were diluted to 1.5 mL during assay termination to achieve the desired glycerol concentration, as indicated in the table. Incubation volume of the fifth set was 0.75 mL, and termination volume was 0.75 mL, and glycerol concentration was 30% thoughout, so that this set served as control for the volume changes.

soluble receptor was not simply due to the effects of glycerol and suggests that it was indeed a property of the interaction of the soluble receptor with follitropin.

It was necessary to incorporate several other controls in this experimental design. Since glycerol has been reported to interfere with precipitation of hCG–receptor complexes (Ascoli, 1983), it was necessary to control for the possible effects of glycerol on PEG precipitation of FSH–receptor complexes. The results are shown in Table I. Specific binding did not differ significantly among these conditions, and unlike hCG–receptor complexes, PEG precipitation of FSH–receptor complexes would appear to be unaffected by glycerol.

Discussion

The testicular FSH receptor has been studied in membrane-bound (Abou-Issa & Reichert, 1976), detergent-solubilized (Abou-Issa & Reichert, 1977; Dias et al., 1981), buffer-solubilized (Dias & Reichert, 1982), and vesicular (Branca et al., 1985) forms. One difficulty during purification has been instability of the binding activity. Membrane-associated receptor retained FSH binding activity from 4 to 24 °C and could be stored at −70 °C without loss of binding

activity, but detergent-solubilized receptor lost all binding activity within hours at 4 °C unless stored in 30% glycerol (Dias et al., 1981). Ascoli (1983) has reported that glycerol interferes with PEG-induced precipitation of complexes during termination of hCG–receptor binding assays. Since PEG is also used to terminate FSH-solubilized receptor binding assays, it was important to determine whether glycerol would affect PEG precipitation of FSH–receptor complexes. Although the nature of glycerol interference with PEG precipitation of hCG–receptor complexes was not described, our findings (Table I) suggest that glycerol does not interfere with FSH–receptor complex precipitation. The assay utilized here, therefore, would appear to be suitable for our studies. The other components of the assay buffer (i.e., detergent, buffer salts, etc.) did not affect the results (results not shown) as they were present in low concentrations and were constant throughout all assays.

Gekko and Timasheff (1981a,b) have shown that glycerol stabilizes proteins by causing them to be preferentially hydrated and this hydration favors native, highly folded structures over denatured, unfolded structures. It was important to determine whether preferential hydration of FSH or the receptor would affect the thermodynamic analysis of the binding reaction. It might be argued that enhanced hydration of the hormone or the receptor would lead to a heat capacity of greater negative magnitude due to increased release of water molecules from protein surfaces following FSH binding to receptor. The detergent-solubilized FSH receptor, prepared as described by Dias et al. (1981), cannot be studied in the absence of glycerol due to its instability, so the effect of glycerol on the thermodynamics of FSH binding was studied by using membrane-bound receptor. The results (Figure 4) indicate that glycerol does affect thermodynamics of FSH binding but does not lead to a more negative ΔC_p. The region in which both the enthalpy and entropy terms contribute favorably to ΔG is between 19 and 25 °C (temperature span of 6 °C, as seen in the earlier work; Andersen et al., 1983) in the absence of glycerol and between 23 and 39 °C (temperature span 16 °C) in the presence of 30% glycerol. Thus, the major effect of glycerol would appear to be on the entropy term; there is a smaller ΔS for binding in the presence of glycerol perhaps due to greater structure in the glycerol–water cosolvent than exists in the aqueous situation. In addition, the change in heat capacity was less negative in the presence of glycerol than in its absence. At 25 °C, ΔC_p was −2.3 kcal mol^{-1} K^{-1} in the absence of glycerol and −0.8 kcal mol^{-1} K^{-1} in 30% glycerol. Here again, the data suggest a more ordered solvent but clearly do not suggest that glycerol can increase the magnitude of ΔC_p. The alternative explanation, that glycerol does not lead to preferential hydration of these proteins (FSH and receptor), would seem less attractive in view of the careful measurements of Gekko and Timasheff (1981a,b).

Having established that glycerol would not compromise binding measurements, thermodynamic studies of FSH binding to soluble receptors were undertaken. Reversibility of FSH binding with solubilized receptors, the first parameter to be measured, differs from that of the membrane-associated receptor in that the former is more uniformly reversible (Figure 1). This may indicate that nonreceptor factors, such as the gonadotropin binding lectins postulated by Calvo and Ryan (1985) or other factors, may have been separated from receptor by solubilization. Whatever the mechanism, the enhanced reversibility for the solubilized system is an important improvement and suggests that thermodynamic analyses are likely to be superior estimates to those obtained for the membrane-bound form of the receptor.

Figure 3 illustrates the relative contributions of enthalpy and entropy to the reaction of FSH with its solubilized bovine testicular receptor. The temperature span in which both enthalpy and entropy contributions to ΔG are favorable is from 14 to 19 °C, which is at lower temperature than for membrane-bound receptors in the presence of glycerol. The heat capacity observed for FSH binding to solubilized receptor is greater than that determined for FSH binding to membrane-bound receptor and is unusually large (Waelbroeck et al., 1979; Formisano et al., 1977). The magnitude of the heat capacity indicates that the hydrophobic effect is indeed the most important force in FSH binding, as was previously postulated when using the membrane–receptor system (Andersen et al., 1983). The increase in magnitude may be due to separation of the receptor from nonreceptor membrane components since other conditions are very similar for the solubilized and membrane-bound receptors in the presence of glycerol. Nonreceptor membrane components such as lectins or enzymes would presumably interact with the hormone–receptor complex at some step subsequent to the initial binding reaction and perhaps cause irreversible binding. Obviating the subsequent interactions allows the initial event to be observed more directly, and the result appears to be an enhancement of the hydrophobic effect.

We propose that the first event is most appropriately described in terms of the hydrophobic effect, as determined by the data presented in this study. We further propose that following formation of this initial "hydrophobically-bound complex" [terminology of Ross and Subramanian (1981)], further events may occur which rely on other types of forces while the receptor is still in the membrane. These events could include nonreceptor processes such as lectin or enzyme interactions but could also be receptor-specific processes such as hydrogen bonds and van der Waals forces. Evidence for participation by these forces includes data which implicate participation by a lectin in binding (Calvo & Ryan, 1985) and the specific inhibitory effects of the Na^+ ion (Andersen & Reichert, 1982). Thus, despite the importance of the hydrophobic effect in the initial phase of the hormone receptor interaction, there is still reason to believe that other forces participate in subsequent steps leading to irreversible binding.

This is one of the first comparisons of the effect of solubilization of a membrane-bound receptor on the thermodynamics of binding. The significance of the study is enhanced because results were obtained with a receptor preparation whose reversibility did not vary appreciably as a function of temperature. The similarity of conclusions obtained from receptor in detergent-solubilized and membrane-bound (Andersen et al., 1983) environments indicates the overwhelming importance of the hydrophobic effect in FSH binding and possibly of the binding of other glycoproteins as well. Nevertheless, enthalpy-dominated events as part of a two-state model should not be ignored, as discussed here. Any attempts to intervene in such reactions may be most effective when both types of forces are manipulated: the hydrophobic effect leading to formation of partially immobilized "hydrophobically-associated" from "individually hydrated" species, followed by hydrogen bonding, van der Waals forces, and/or electrostatic interactions leading to an "interacting complex", as defined by Ross and Subramanian (1981).

Registry No. FSH, 9002-68-0.

REFERENCES

Abou-Issa, H., & Reichert, L. E., Jr. (1976) *J. Biol. Chem. 251*, 3326–3337.

Abou-Issa, H., & Reichert, L. E., Jr. (1977) *J. Biol. Chem. 252*, 4166–4174.
Andersen, T. T., & Reichert, L. E., Jr. (1982) *J. Biol. Chem. 257*, 11551–11557.
Andersen, T. T., Curatolo, L. M., & Reichert, L. E., Jr. (1983) *Mol. Cell. Endocrinol. 33*, 37–52.
Ascoli, M. (1983) *Endocrinology (Baltimore) 113*, 2129–2134.
Branca, A. A., Sluss, P. M., Smith, R. A., & Reichert, L. E., Jr. (1985) *J. Biol. Chem. 260*, 9988–9993.
Calvo, F. O., & Ryan, R. J. (1985) *Biochemistry 24*, 1953–1959.
Dattatreyamurty, B., Schneyer, A. L., & Reichert, L. E., Jr. (1986) *J. Biol. Chem. 261*, 13104–13113.
Dias, J. A., & Reichert, L. E., Jr. (1982) *J. Biol. Chem. 257*, 613–620.
Dias, J. A., Huston, J. S., & Reichert, L. E., Jr. (1981) *Endocrinology (Baltimore) 109*, 736–742.
Dias, J. A., Treble, D. H., & Reichert, L. E., Jr. (1983) *Endocrinology (Baltimore) 113*, 2029–2034.
Formisano, S., Johnson, M. L., & Edelhoch, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3340–3344.
Gekko, K., & Timasheff, S. N. (1981a) *Biochemistry 20*, 4667–4676.
Gekko, K., & Timasheff, S. N. (1981b) *Biochemistry 20*, 4677–4686.
Greenwood, F. C., Hunter, W. M., & Glover, J. S. (1963) *Biochem. J. 89*, 114–123.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220–239.
Pierce, J. G., & Parsons, T. F. (1981) *Annu. Rev. Biochem. 50*, 465–495.
Reichert, L. E., Jr., Andersen, T. T., Dias, J. A., Fletcher, P. W., Sluss, P. M., O'Neill, W. C., & Smith, R. A. (1984) in *Hormones in Growth and Reproduction* (Saxena, B. J., Catt, K. J., Birnbaumer, L., & Martini, L., Eds.) pp 87–101, Raven Press, New York.
Ross, P. D., & Subramanian, S. (1981) *Biochemistry 20*, 3096–3102.
Schaffner, W., & Weissman, C. (1973) *Anal. Biochem. 56*, 502–514.
Schneyer, A. L., Sluss, P. M., Dattatreyamurty, B., & Reichert, L. E., Jr. (1985) *Endocrinology (Baltimore) 119*, 1446–1453.
Waelbroeck, M., Van Obberghen, E., & De Meyts, P. (1979) *J. Biol. Chem. 254*, 7736–7740.

Mechanism of Action of *Escherichia coli* Endonuclease III†

Yoke W. Kow and Susan S. Wallace*
Department of Microbiology and Immunology, New York Medical College, Valhalla, New York 10595
Received March 20, 1987; Revised Manuscript Received June 23, 1987

ABSTRACT: Endonuclease III isolated from *Escherichia coli* has been shown to have both *N*-glycosylase and apurinic/apyrimidinic (AP) endonuclease activities. A nicking assay was used to show that the enzyme exhibited a preference for form I DNA when DNA containing thymine glycol was used as a substrate. This preference was reduced or eliminated either when the DNA was relaxed or when the type of damage was altered to urea residues or AP sites. The combined *N*-glycosylase/AP endonuclease activity was at least 10-fold higher than the AP endonuclease activity alone when urea-containing DNA was used as a substrate as compared to AP DNA. When DNA containing thymine glycol was used as a substrate, the combined *N*-glycosylase/AP endonuclease activity was about 2-fold higher than the AP endonuclease activity. Yet, when DNA containing thymine glycol or urea was used as substrate, no apurinic sites remained. Furthermore, magnesium selectively inhibited endonuclease III activity when AP DNA was used as a substrate but had no effect when DNA containing either urea or thymine glycol was used as substrate. These data suggest that both the *N*-glycosylase and AP endonuclease activities of endonuclease III reside on the same molecule or are in very tight association and that these activities act in concert, with the *N*-glycosylase reaction preceding the AP endonuclease reaction.

The base excision repair pathway for the removal of modified DNA bases involves two types of enzymes, DNA *N*-glycosylases and apurinic/apyrimidinic (AP) endonucleases. The sequential action of these enzymatic activities removes the modified base, leaving a nick adjacent to an abasic site on the DNA molecule.

Endonuclease III from *Escherichia coli* has been shown to be an AP endonuclease with an associated *N*-glycosylase activity (Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984). Although it has not been unambiguously established, both activities appear to reside on the same molecule having a native molecular weight of about 25 000 (Katcher & Wallace, 1983; Breimer & Lindahl, 1984). The associated glycosylase activity recognizes a spectrum of thymine ring saturation or fragmentation products including thymine glycols (Armel et al., 1977; Gates & Linn, 1977; Demple & Linn, 1980; Katcher & Wallace, 1983; Breimer & Lindahl, 1984), 5,6-dihydrothymine (Demple & Linn, 1980; Ide, Kow, and Wallace, unpublished data), urea (Breimer & Lindahl, 1980; Katcher & Wallace, 1983), 5-hydroxy-5-methylhydantoin (Breimer & Lindahl, 1984, 1985), and methyltartronylurea (Breimer & Lindahl, 1984). The enzyme also recognizes some unknown cytosine radiolysis product(s) (Doetsch et al., 1986; Weiss & Duker, 1986).

Most studies undertaken with repair endonucleases and glycosylases have focused on substrate specificity, subunit structure, the nature of the nick produced, and other enzymatic reaction parameters. With the exception of the uracil DNA

† This work was supported by National Institutes of Health Grants CA33657 and GM37216.
* Author to whom correspondence should be addressed.

0006-2960/87/0426-8200$01.50/0