Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 2263–2266, April 1983
Cell Biology

Binding of apotransferrin to K562 cells: Explanation of the transferrin cycle

(receptor-mediated endocytosis/recycling/pH-dependent binding)

RICHARD D. KLAUSNER, GILBERT ASHWELL, JOS VAN RENSWOUDE, JOE B. HARFORD, AND KENNETH R. BRIDGES*

Laboratory of Biochemistry and Metabolism, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

Contributed by Gilbert Ashwell, January 24, 1983

ABSTRACT **The binding of apotransferrin to the transferrin receptor on the surface of human leukemic K562 cells was found to be significantly less tight than that of the holoprotein, diferric transferrin. The finding that both ligands displayed linear Scatchard plots with similar receptor number (≈150,000 per cell) and mutually inhibit each other's binding suggested that they bind to the same receptor. Both the dissociation and association rate of apotransferrin were markedly increased (28-fold and 15-fold, respectively) at pH 7.2 compared to pH 4.8. Using the values of these binding parameters, we propose a mechanism to account for the recycling of transferrin subsequent to internalization and residence within an acidic nonlysosomal organelle where iron is removed.**

A wide variety of molecules gain entry into cells by receptor-mediated endocytosis (1, 2). Diverse ligands, including low density lipoproteins, asialoglycoproteins, epidermal growth factor, α_2 macroglobulin, lysosomal enzymes, and certain hormones, toxins, and viruses traverse strikingly similar, if not identical, pathways. Binding to specific cell-surface receptors is followed by internalization involving specialized regions of the plasma membrane, the coated pits. Between their internalization and their ultimate fate, which is often lysosomal degradation, appropriately tagged ligands are visible by electron microscopy within a number of morphologically varied intracellular vesicles. We have examined this pathway for the receptor-mediated endocytosis of asialoglycoproteins in hepatocytes and have shown that the receptor enters the cell as a complex with its ligand, whereupon the latter subsequently dissociates and is degraded (3, 4). In contrast to the ligand, the receptor is reutilized (5, 6). We have presented evidence that the intracellular dissociation of asialoglycoproteins is facilitated by encounter with an acidic environment prior to delivery to lysosomes (4, 7), a step that we believe to be critical in receptor reutilization. There is growing evidence that a number of other ligands are similarly exposed to nonlysosomal environments of low pH in their movement through the cell (8–11). This suggests that an acidic endocytic vesicle may participate in the intracellular transit of many ligands. As with asialoglycoproteins, a number of ligands interact with their respective receptors in a highly pH-dependent fashion, and many of these receptors have been shown to be reutilized (1).

A notable exception to this generalized picture of receptor-mediated endocytosis involves the iron-binding protein transferrin (reviewed in ref. 12). Diferric transferrin enters the cell bound to its specific receptor, but internalization of the receptor-bound transferrin does not result in transferrin degradation (13–15). Instead, both the receptor and the apoprotein ligand are returned to the cell's exterior with retention of iron within the cell. Although coated pits (16) and an acidic, nonlysosomal microenvironment (9) have been shown to be involved in the transferrin pathway, transferrin does not enter lysosomes (9) and thereby escapes degradation. There has been no satisfactory explanation for this distinctive behavior of transferrin. Here we have examined the pH sensitivity of the interaction of the transferrin receptor with both diferric transferrin and apotransferrin. The characteristics of these interactions appear to explain the transferrin cycle.

MATERIALS AND METHODS

Cells. Human erythroleukemia K562 cells (17) were grown in RPMI 1640 medium (National Institutes of Health Media Unit) with 10 mM Hepes and 10% heat-inactivated fetal bovine serum (GIBCO). Cells were maintained at densities of 2–5 × 10^5 cells per ml, and all experiments were performed with cells that were collected at a density of 5 × 10^5 cells per ml. Prior to use, cells were washed at 4°C with RPMI 1640 medium containing 0.1% Norit-treated bovine serum albumin. The cells were then suspended (0.5–2 × 10^7 cells per ml) in the wash medium containing 25 mM sodium acetate and adjusted to the desired pH.

Ligands. Human transferrin (Calbiochem) was radiolabeled (to a specific activity of 300–900 cpm/ng) with ^{125}I with immobilized Enzymobead lactoperoxidase-glucose oxidase (Bio-Rad) according to the manufacturer's instructions. By using previously published procedures (15), ^{125}I-labeled transferrin (^{125}I-transferrin) was saturated with iron to produce diferric ^{125}I-transferrin, or the iron was removed to yield ^{125}I-labeled apotransferrin (^{125}I-apotransferrin). Unlabeled diferric and apotransferrin were prepared in analogous fashion. Diferric transferrin was stored at 4°C as a solution in 150 mM NaCl/20 mM Tris·HCl, pH 7.4. Apotransferrin was similarly stored except that 10 μM deferoxamine mesylate (CIBA Pharmaceutical) was included.

Binding Assays. Freshly prepared deferoxamine was added to washed K562 cells at 4°C to a final concentration of 25 μM. The appropriate radiolabeled ligand was added to the cell suspension; after 90 min on ice, 0.1 ml of the incubation mixture was layered onto dibutyl phthalate (150 μl) in a 400-μl Eppendorf tube and centrifuged for 10 sec at 8,000 × *g*. The tips of the tubes containing the cell pellets were excised, and the cell-associated radioactivity was determined in a Beckman 5500 gamma spectrometer. Nonspecific binding was assessed by addition of a 100-fold excess of unlabeled ligand 5 min prior to

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* Present address: Department of Hematology, Brigham and Women's Hospital, Boston, MA.

radiolabeled ligand. All assays were performed in the RPMI/albumin/acetate mixture.

RESULTS

The binding of apotransferrin and diferric transferrin to K562 cells at 4°C were compared. Equilibrium binding data were subjected to Scatchard analyses, resulting in typical binding isotherms (Fig. 1). Apotransferrin displayed an apparent K_d of 4.8×10^{-8} M for approximately 1.5×10^5 binding sites per cell (Fig. 1*A*). The apparent K_d for diferric transferrin calculated from the data in Fig. 1*B* was some 95% lower at 2.1×10^{-9} M. The receptor number was unchanged at about 1.5×10^5 sites per cell. Evidence that the same receptor sites were involved was provided by the demonstration that an excess of either unlabeled ligand inhibited the binding of both radiolabeled tracer ligands (data not shown).

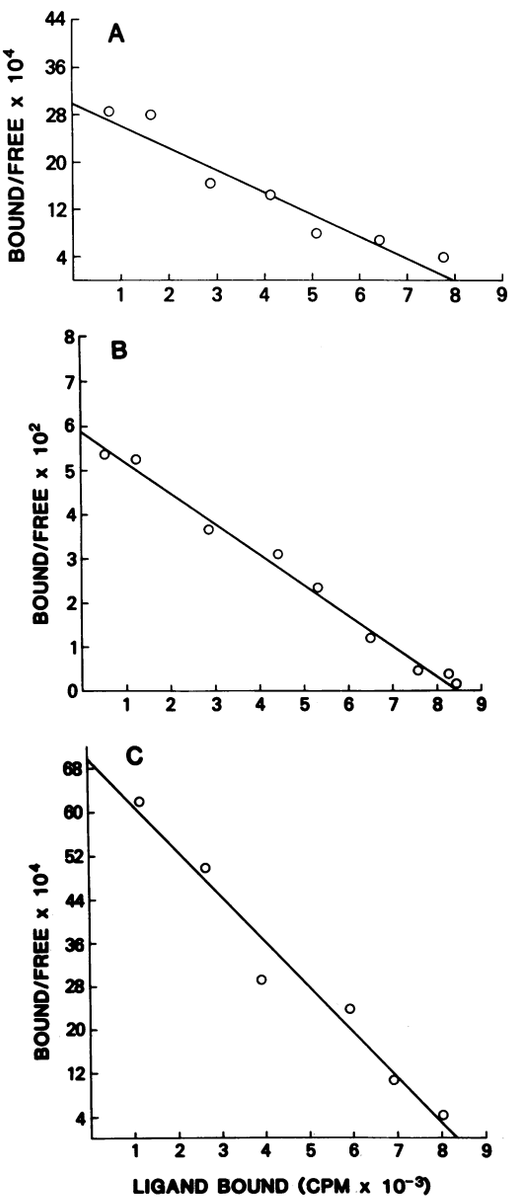

FIG. 1. Scatchard analyses of binding of apotransferrin and diferric transferrin to K562 cells at 4°C. Binding assays were performed with 5×10^5 cells and ^{125}I-labeled ligands with a specific activity of 800 cpm/ng. Data were plotted according to Scatchard (18). (*A*) Binding of apotransferrin at pH 7.2. (*B*) Binding of diferric transferrin at pH 7.2. (*C*) Binding of apotransferrin at pH 4.8. Nonspecific binding determined in the presence of a 100-fold excess of unlabeled diferric transferrin has been subtracted. The data shown are derived from one experiment. We performed a total of 6–8 binding experiments per type of ligand at both pH 4.8 and pH 7.2 and obtained the following average dissociation constants (± standard error of the mean): for diferric transferrin at pH 7.2, $K_d = 1.9 \pm 1.2 \times 10^{-9}$ M; for apotransferrin, $K_d = 2.1 \pm 1.1 \times 10^{-8}$ M (at pH 4.8) and $K_d = 4.8 \pm 2.2 \times 10^{-8}$ M (at pH 7.2).

We have shown that transferrin internalized by K562 cells resides in a nonlysosomal compartment at a pH of about 5.5 (9). Many ligands, including asialoglycoproteins, dissociate from their respective receptors at or below pH 6. This is not the case for diferric transferrin. Fig. 2 demonstrates that even pH 5 was insufficiently low to effect the dissociation of diferric transferrin from its receptor. In this experiment, cells were saturated with ligand at pH 7.2, washed, and resuspended in medium adjusted to the indicated pH. After 5 min at 4°C, the cells were centrifuged through dibutyl phthalate, and the amount of cell-associated ligand was determined.

As noted above, iron is removed from transferrin during its transit through the cell. It is conceivable that the removal of iron alters the pH dependence of the receptor–ligand interaction. When we attempted to assess the pH dependence of apotransferrin dissociation by the washing procedure described above for diferric transferrin, negligible binding was found at neutral pH. On the premise that apotransferrin might dissociate rapidly from its receptor at neutral pH, we replaced the relatively slow washing procedure (≈4 min) by the direct and rapid centrifugation of cells through dibutyl phthalate. With the latter method, the binding of apotransferrin to its receptor at neutral pH was readily measurable. Cells were incubated with a saturating concentration of ligand at 4°C at pH 7.2. After 45 min, predetermined amounts of 1 M HCl were added to obtain the desired final pH. After an additional 5 min at the new pH, cell-associated ligand was determined by rapid centrifu-

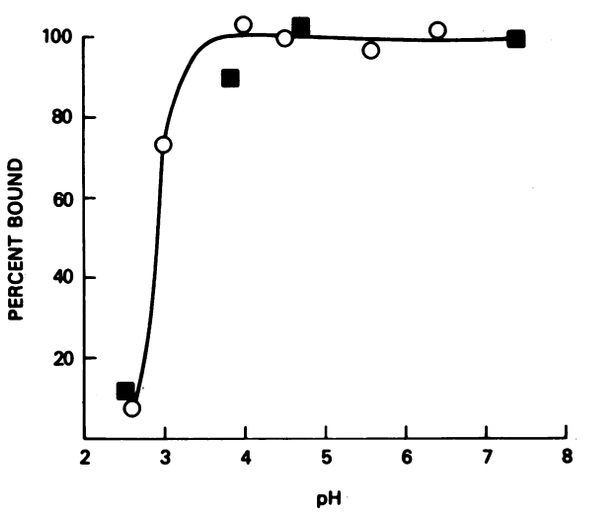

FIG. 2. Dependence of ligand binding on pH. K562 cells (10^7 cells per ml) were incubated with diferric ^{125}I-transferrin (10 μg/ml) for 45 min at 4°C. Cells were washed with incubation medium and suspended in medium at the indicated pH. After an additional 5 min on ice, the cells were centrifuged through dibutyl phthalate. Data for cell-associated diferric transferrin (○) are expressed as a percentage of the value at pH 7.2. In experiments with ^{125}I-apotransferrin (100 μg/ml), 25 μM deferoxamine was included, and the wash and resuspension steps were omitted. Instead, predetermined amounts of 1 M HCl were added at 45 min to arrive at the indicated pH. After an additional 5 min on ice, cells were centrifuged through dibutyl phthalate. Data for apotransferrin binding (■) are expressed as a percentage of the value at pH 7.2. In both cases nonspecific binding was subtracted in the calculations. Of note is the fact that nonspecific binding goes up dramatically below pH 4.5.

gation through dibutyl phthalate. With this procedure, the dependence of apotransferrin binding on pH did not appear to differ from that of diferric transferrin (Fig. 2). Indeed, a Scatchard analysis revealed that the equilibrium binding of apotransferrin at pH 4.8 was characterized by a K_d of 2.1×10^{-8} M (Fig. 1C)—approximately half that determined at pH 7.2 (Fig. 1A). Thus, while apotransferrin binds to the receptor at both pH 4.8 and pH 7.2, binding is somewhat better at the lower pH.

Subsequent to endocytosis of the receptor–diferric transferrin complex, the iron is removed. The probability of the resultant apotransferrin remaining bound to the receptor is a function of the dissociation rate within the acidic (≈pH 5) endocytic compartment. To determine this rate, cells at 4°C were saturated with ^{125}I-apotransferrin at pH 4.8 prior to washing and resuspension in ligand-free medium at the same pH. At intervals, aliquots were removed and centrifuged through dibutyl phthalate. The $t_{1/2}$ for dissociation at pH 4.8 was calculated to be ≈17 min (Fig. 3). Alternatively, after loading at pH 4.8, the cells were washed and resuspended in ligand-free medium at pH 7.2 either immediately or after 35 min of dissociation at pH 4.8. In both instances, dissociation at pH 7.2 was markedly (≈28-fold) more rapid with a $t_{1/2}$ of ≈40 sec. Because the equilibrium dissociation constants at the two pHs differed by only 2-fold, these data predicted that the association rates of apotransferrin must also differ at these pH values. This was found to be the case. The K562 cells at 4°C were exposed to a subsaturating concentration of ^{125}I-apotransferrin at pH 4.8 and at pH 7.2, and the cell-associated ligand was determined at various times. It is clear from Fig. 4 that much less time is required to reach plateau binding at pH 7.2 than at pH 4.8. Computer-assisted analyses of these curves yielded association rate constants of 0.09 ± 0.01 min^{-1} for pH 4.8 and 1.3 ± 0.2 min^{-1} for pH 7.2, assuming a first-order interaction. Thus, the "on-rate" is 15-fold greater at pH 7.2.

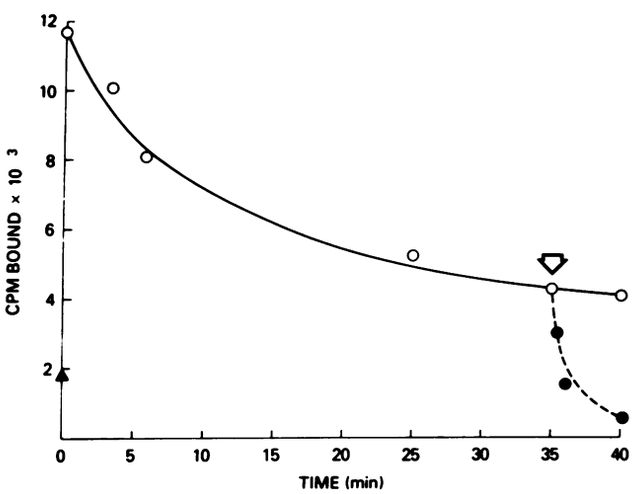

FIG. 3. Dissociation of apotransferrin from K562 cells. Cells (1.5×10^7 cells per ml) were incubated with ^{125}I-apotransferrin (1 μg/ml) for 45 min at 4°C in medium at pH 4.8. The cells were centrifuged at 1,500 × *g* for 5 min and resuspended in medium at pH 4.8 (○). Incubation was continued at 4°C, at the indicated time, aliquots were centrifuged through dibutyl phthalate, and the amount of cell-associated radioactivity was determined. At 35 min (arrow), a predetermined amount of 1 M NaOH was added to a portion of the cells to yield a pH of 7.2. Aliquots from this sample were similarly assayed (●). When the cells were washed and resuspended in medium at pH 7.2 at time zero, most of the ligand was dissociated (▲) within the time required for washing (4 min). Values for nonspecific binding have been subtracted.

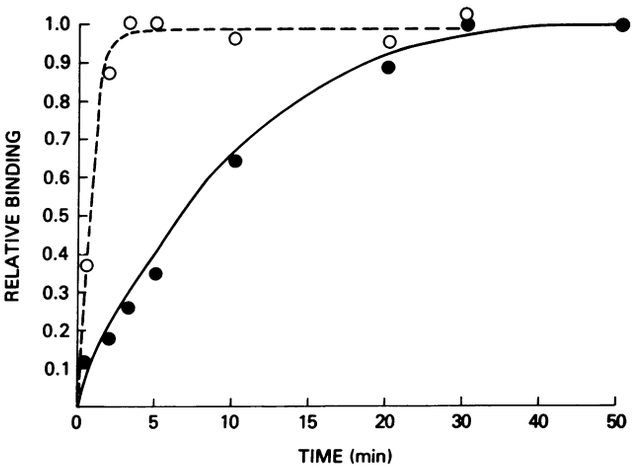

FIG. 4. Association of apotransferrin to K562 cells. Cells (1×10^7 cells per ml) were incubated with ^{125}I-apotransferrin (0.015 μg/ml) in medium at pH 7.2 (○) or pH 4.8 (●). At the indicated times, a sample was centrifuged through dibutyl phthalate, and the amount of cell-associated radioactivity was determined. Data are expressed in arbitrary units to indicate relative binding because only 40% as much ligand became cell-associated at pH 7.2 as at pH 4.8.

DISCUSSION

Subsequent to internalization of diferric transferrin, iron is removed and transferred to cytosolic ferritin (15). The intracellular transferrin resides in an acidic (pH 5–5.8) microenvironment (9). Apotransferrin returns to the cell surface, is released from the cell, and is available for additional iron binding and endocytosis. The data presented here suggest that the low pH of the compartment in which transferrin is present facilitates this cycle. The equilibrium binding of apotransferrin to the receptor is less tight than that of diferric transferrin. Whereas the K_d for apotransferrin is minimally changed by pH (2-fold), the rate of association and, perhaps more importantly, the rate of dissociation are much more dramatically affected by pH.

The dissociation of even one molecule of apotransferrin within a small endocytic vesicle (with a volume of ≈10^{-17} liter) would result in a concentration of apotransferrin that is high relative to the K_d. Thus, apotransferrin may be expected to remain receptor-bound in such a vesicle. Moreover, the maintenance of the receptor-apotransferrin interaction would be favored by the relatively slow dissociation rate at pH 4.8. As the vesicle containing the receptor–apotransferrin complex fuses with the plasma membrane, two things happen. The complex is exposed to the neutral pH of the extracellular milieu, and the ligand is greatly diluted. The former condition would result in a dramatic increase in the dissociation rate, and the latter renders rebinding improbable. Thus, the apotransferrin is released from the receptor and leaves the cell. It should be noted that the parameter values calculated here will probably deviate from the physiological rate and equilibrium constants because they had to be determined at 4°C rather than 37°C. At 37°C, dissociation may occur more readily. However, the entire transferrin cycle in K562 cells takes only 4–5 min (15). Moreover, it is not known at what point in the process iron is removed. Thus a 5- to 10-fold increase in the dissociation rate of apotransferrin at 37°C may well be compatible with stability of the complex within the endocytic vesicle. The findings reported in this paper serve to explain how the general pathway of receptor-mediated endocytosis is utilized; by differing in their response to low pH, various receptor–ligand complexes may be sorted and directed within the cell.

During the preparation of this manuscript, a study by Dautry-Varsat *et al.* (19) was kindly made available to us. Although there are differences in the equilibrium binding constants for apotransferrin at neutral pH, they present a model for the transferrin cycle consistent with that presented here.

J.V.R. gratefully acknowledges the financial support from the Niels–Stensen Stichting, the Netherlands, and the Netherlands Organization for the Advancement of Pure Scientific Research (Z.W.O.).

1. Goldstein, J. L., Anderson, R. G. W. & Brown, M. S. (1979) *Nature (London)* **279,** 679–685.
2. Pastan, I. & Willingham, M. C. (1981) *Annu. Rev. Physiol.* **52,** 239–250.
3. Bridges, K., Harford, J., Ashwell, G. & Klausner, R. D. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 350–354.
4. Harford, J., Bridges, K., Ashwell, G. & Klausner, R. D. (1983) *J. Biol. Chem.* **258,** 3191–3197.
5. Steer, C. J. & Ashwell, G. (1980) *J. Biol. Chem.* **255,** 3008–3013.
6. Tolleshaug, H. (1981) *Int. J. Biochem.* **13,** 45–51.
7. Harford, J., Wolkoff, A. W., Ashwell, G. & Klausner, R. D. (1983) *J. Cell Biol.*, in press.
8. Tycko, B. & Maxfield, F. R. (1982) *Cell* **28,** 643–651.
9. van Renswoude, J., Bridges, K., Harford, J. & Klausner, R. D. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 6186–6190.
10. Maxfield, F. R. (1982) *J. Cell. Biol.* **95,** 676–681.
11. Marsh, M., Wellsteed, J., Kern, H., Harms, E. & Helenius, A. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 5297–5301.
12. Aisen, P. & Listowsky, I. (1980) *Annu. Rev. Biochem.* **49,** 357–393.
13. Karin, M. & Mintz, M. (1981) *J. Biol. Chem.* **256,** 3245–3252.
14. Octave, J.-N., Schneider, Y.-J., Crichter, R. R. & Trouet, A. (1981) *Eur. J. Biochem.* **115,** 611–618.
15. Klausner, R. D., van Renswoude, J., Kempf, C., Ashwell, G., Dean, A., Schechter, A. N. & Bridges, K. R. (1983) *J. Biol. Chem.*, in press.
16. Pearse, B. M. F. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 451–455.
17. Lozzio, C. B. & Lozzio, B. B. (1975) *Blood* **45,** 321–334.
18. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51,** 660–672.
19. Dautry-Varsat, A., Ciechanover, A. & Lodish, H. F. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2258–2262.