Transferrin and the Transferrin Cycle in Belgrade Rat Reticulocytes*

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Belgrade rats have an autosomal recessive anemia with hypochromia and microcytosis. Iron uptake into reticulocytes is ~20% of normal, but transferrin uptake is unimpaired. We have systematically compared the transferrin cycle in Belgrade versus normal reticulocytes to locate the defect more precisely. Belgrade transferrin was functionally normal as purified transferrin or whole plasma. Transferrin affinity of Belgrade receptors was indistinguishable from normal, but Belgrade reticulocytes had twice as many receptors. Belgrade transferrin endocytosis was 1.5 times faster than normal, whereas exocytosis is about twice as fast. Initially Belgrade reticulocytes internalize iron at an unimpaired rate, but they lag behind normal by 5 min. During reincubation, they release 25–33% of iron taken up during a 30-min preincubation, whereas normal cells do not lose a detectable fraction. Unexpectedly, transferrin cycle time was unchanged. Hence another kinetic step of the cycle is slower, compensating for increases in Belgrade endocytosis and exocytosis. After one cycle, Belgrade reticulocytes retain only half of the iron that entered, but over 80% of iron entering normal cells remains within. Iron unloading is ineffective inside the Belgrade vesicle; 85% of iron that entered on transferrin returned to the medium after exocytosis, whereas only 45% of iron entering normal reticulocytes exits. Ineffective utilization of iron in or near Belgrade endosomes accounts for the Belgrade defect.

The Belgrade rat (gene symbol b) has a microcytic, hypochromic anemia inherited in an autosomal recessive manner (Sladic-Simic et al., 1966). Although serum iron is elevated (Sladic-Simic et al., 1969), iron uptake into Belgrade reticulocytes is only about 20% of normal (Edwards et al., 1978). Studies of globin production by reticulocytes demonstrate balanced α and β chain synthesis, although total globin synthesis is only about half-normal (Edwards et al., 1978). The Belgrade defect appears to be present from the earliest stages of red cell development (Pavlovic-Kentner et al., 1989).

Receptor-mediated endocytosis is the mechanism by which iron is normally delivered to reticulocytes (for reviews see Morgan, 1981; Seligman, 1983; Rapoport, 1986). Iron is carried in the plasma by the protein transferrin (Tf). Iron–Tf binds to transferrin receptor (TfR), which triggers internalization into an endocytic vesicle. The Tf–TfR complex recycles to the cell surface, whereas internalized iron is released into the cytoplasm and how iron leaves the vesicle to reach the mitochondria where Fe²⁺ is inserted into protoporphyrin by ferrochelatase remains uncertain. Reduction of Fe³⁺ to Fe²⁺ is required for exit through the plasma membrane (Nunez et al., 1990).

We have shown previously that the Belgrade defect occurs in the delivery of iron through the Tf cycle (Garrick et al., 1988b, 1990a, 1991). When iron is delivered by Fe–SIH, a synthetic iron chelate that bypasses the TfR, iron incorporation into heme is stimulated considerably. Proporphyrin synthesis is normal in Belgrade reticulocytes when Fe–SIH is used to deliver iron, indicating that the proporphyrin synthesis pathway is not defective in Belgrade reticulocytes and that substantial ferrochelatase activity is present. Reduction of Fe³⁺ to Fe²⁺ must also be occurring.

In the present study we have systematically examined the Tf cycle to narrow the location of the Belgrade defect. Emphasis is on comparisons rather than determining absolute values for parameters, because we are trying to account for a 5-fold decrease in iron incorporation by Belgrade versus normal reticulocytes. It appears that substantial transferrin receptor activity is present in Belgrade reticulocytes. A preliminary report (Garrick et al., 1988a) of the Tf analyses has already been made. We also show that Tf turnover is normal in Belgrade reticulocytes, which is consistent with the Tf analyses (Garrick et al., 1988a). We are therefore able to demonstrate that Belgrade reticulocytes deliver iron normally to normal rats.

MATERIALS AND METHODS

Animals

Normal rats (+/b or +/+), where + is either + or b, were bled on days 7, 5, and 3 preceding the experiments to induce a reticulocytosis.
Transferrin Cycle in the Belgrade Rat

of 15–20%. Calculated iron loss was replaced by an intraperitoneal injection of iron dextran. Belgrade rats had a naturally occurring reticulocytosis of about 15–20%. Rats were bled by retro-orbital sinus puncture after ether anesthesia. The State University of New York Institutional Animal Care and Use Committee reviewed and approved all procedures on animals.

**Tf**

Rat Tf was usually purchased from Pel-Freez Biologicals (Rogers, AR), but for certain experiments (below) we used whole serum or Tf purified by us. Tf was depleted of iron and loaded with ¹⁵⁸Fe using the NTA method as described (Garrick et al., 1991). Iodination was performed as in Garrick et al. (1983). Tf protein concentration was measured by the bicinchoninic acid method (Smith et al., 1985). An aliquot was counted for specific radioactivity and ¹⁵⁸Fe.*₁⁄₂ Radioactivity. During each experiment, we tested for linear incorporation of iron into hemoglobin over a 60-min incubation with this doubly labeled Tf and normal (⁺/⁻ or +/⁻) reticulocytes. Linearity indicated that the cells and Tf behaved physiologically so ¹⁵⁸I incorporation accurately represents Tf kinetics in parallel incubations.

**Serum**

Serum from mutant rats was collected and sterilized by passing through a Gelman 0.25-μm filter. Tf in the serum was stripped of iron by dialysis twice against 0.1 M sodium acetate-citrate, pH 4.5, then once against PBS. Iron loading of Tf in the serum with ¹⁵⁸Fe was done using the NTA method as described (Garrick et al., 1993). Serum iron and unincorporated iron binding capacity were measured using the ferrozine method (Sigma). Iron-loaded normal rat serum was prepared and assayed similarly. Total iron binding capacity and percentage iron saturation were then calculated and matched for the two preparations.

**Tf Purification**

Plasma from Belgrade rats was pooled and Tf purified using the modifications below of the method of Okada et al. (1979). Tf from normal rats was similarly purified. After ammonium sulfate precipitation, redissolved protein was dialyzed against 50 mM Tris-HCl, pH 8.5, and concentrated by ultrafiltration. The sample was then placed on a 1.5 × 80-cm column of DEAE-cellulose (DE-52, Whatman, Maidstone, Kent, United Kingdom) equilibrated with 50 mM Tris-HCl, pH 8.5. Tf was eluted using a linear gradient of 0–500 mM NaCl added to the Tris buffer. The pink-colored eluate was pooled, concentrated by ultrafiltration, dialyzed versus the Tris buffer, and applied to a column of DEAE-cellulose. The column was eluted with a linear gradient of 0–100 mM NaCl added to the Tris buffer. Purification was repeated using the second gradient until A₂₈₀/A₂₆₀ was 1.2. Purity was determined by SDS-polyacrylamide gel electrophoresis using the system of Laemmli (1970). Tf purity was about 97%. Tf was loaded with ¹⁵⁸Fe using the NTA method (Garrick et al., 1991). Percentage saturation was calculated after counting the preparation in a γ counter to determine the ratio pmol iron/mmol Tf (2:1 = 100%).

**Incubations**

**Incorporation**—Heparinized reticulocyte rich blood (referred to as reticulocytes hereafter for convenience) was collected from normal (+/⁻) and Belgrade (⁺/⁺) rats. Reticulocytes were washed, incubated, and lysed according to Garrick et al. (1993) except that 150 mM NaCl, 5 mM KCl, and 5 mM MgCl₂ replaced HBSS. Both ¹⁵⁸Fe and ¹²⁵I were determined.

**Purified Tf and Serum**—Washed +/⁻ reticulocytes were resuspended in ¹⁵⁸Fe serum or ¹⁵⁸Fe-Tf plus incubation medium at a hematocrit of 25% and were incubated at 37°C. Aliquots were sampled at 15, 30, and 60 min and placed into ice-cold PBS containing 1 mg/ml Pronase to remove surface-bound label. After 20 min on ice, cells were washed three times with PBST at 4°C and lysed with 5 mM Tris-HCl, pH 8.6. Both ¹⁵⁸Fe and ¹²⁵I counts/min were determined.

**Exocytosis**—To measure the rate of exocytosis, we incubated washed reticulocytes with 1 μM ¹⁵⁸Fe-Tf in incubation medium at a hematocrit of 25% at 37°C. Aliquots were removed at selected intervals to ice-cold PBS containing 1 mg/ml Pronase to remove surface-bound label. After 20 min on ice, cells were washed three times with PBST at 4°C and lysed with 5 mM Tris-HCl, pH 8.6. Both ¹⁵⁸Fe and ¹²⁵I counts/min were determined.

**Endocytosis**—To measure the rate of endocytosis, we incubated washed reticulocytes with 1 μM ¹⁵⁸Fe-Tf for 30 min at a hematocrit of 25% and 37°C. Cells were then treated with Pronase as above and washed three times with ice-cold PBS at 4°C. Cells were placed into prewarmed incubation medium at a hematocrit of 25% with 1 μM unlabeled diferric reticulin and reincubated at 37°C. Aliquots were taken at selected intervals, placed into ice-cold PBS, and centrifuged. Both cells and supernatants were counted for ¹⁵⁸Fe and ¹²⁵I.

**Single Cycle**—Washed reticulocytes from b/b rats and their normal litter mates were preincubated with 1 μM (or in one experiment 5 μM) ¹⁵⁸Fe-Tf in incubation medium for 60 min at 0°C; washed reticulocytes were then loaded with ¹⁵⁸Fe and reincubated for 60 min at 0°C to load surface Tf with Tf. Unbound Tf was removed by washing with HBSS three times at 4°C. Cells were then reincubated in prewarmed incubation medium plus 1 μM nonradioactive diferric Tf at 37°C and a hematocrit of 25%. Aliquots were removed to ice-cooled PBS at 37°C. Cells were then extracted with 1 mg/ml Pronase in PBS for 20 min on ice to remove surface Tf and iron-Tf. Both halves were then spun through dibutylphthalate in Eppendorf tubes. Cell pellets and supernatants were counted for ¹⁵⁸Fe and ¹²⁵I. The pellet of the Pronase treated half yielded internal cellular counts. The supernatant of the untreated half yielded counts in the medium, and the pellet yielded total cellular counts. Surface counts equal total minus internal.

**Sample Analysis and Data Reduction**

Heme was extracted by the oxalic acid-acetone method (Garrick et al., 1975). Cellular and heme ⁹⁹Fe counts/min and ¹²⁵I counts/min were measured in an LKB Compugamma counter. RNA was determined as described previously (Edwards et al., 1978); because of uncertainties and imprecision in counting reticulocytes, comparisons between Belgrade and control rats were based on RNA denominators. Data on rates of incorporation for Belgrade versus normal Tf were analyzed on an AT compatible computer using the program STATA (Computing Resource Center, Los Angeles, CA). Data from Scatchard analysis were reduced using LIGAND (MSDOS version) from Munson and Rodbard (1980). Data from endocytosis, exocytosis, and single cycle experiments were reduced using both STATA and MINSQ (Micromath, Salt Lake City, UT).

Data for endocytosis studies were reduced to counts/min/μg RNA, then fitted to an exponential model that predicts the maximum concentration of Tf bound; the data were then normalized as 1 - (endocytosed/maximum) to estimate a rate constant. Similarly, data for exocytosis studies were reduced to counts/min/μg RNA and then fitted to an exponential model that predicts the initial concentration of Tf within the cells; the data were then normalized as (not yet exocytosed)/(initial concentration) to estimate a rate constant. Both approaches were applied to the data for +/⁻ and b/b animals individually and in combination with MINSQ reporting parameters that estimate the S.D. and appraising whether residuals are normally distributed. The ratio of S.D. for +/⁻ and b/b animals individually and in combination with MINSQ reporting parameters that estimate the S.D. and appraising whether residuals are normally distributed. The ratio of S.D. for +/⁻ or b/b to S.D. for the combined data was treated as being distributed like Student’s distribution for hypothesis testing treating p values as distributed like Student’s distribution for hypothesis testing treating p as small as possible.

Data reduction for single cycle experiments was based on a modification* of the usual dual exponential model. The analysis

\[ A \sim B \sim C \] (Reaction 1)

is the path of interest, where k₁ = rate constant for endocytosis; k₂ = rate constant for exocytosis; then

\[ A = A₀ \cdot e^{-k₂ t} \] (Eq. 1)

\[ B = \left( k₂ (B₀ + k₁) \right) \cdot (A₀ - A₀ \cdot e^{-k₂ t}) \] (Eq. 2)
yielded estimates for rate constants for both endocytosis and exocytosis. MINSQ reported parameters similar to those for analysis of endocytosis only (or exocytosis only) with statistical hypotheses tested as above.

RESULTS

Iron and Tf Incorporation—Edwards et al. (1978) have shown that Belgrade reticulocytes incorporate iron from Tf at about 20% of the rate of cells from normal rats although iron incorporation into the two types of cells for periods of 5 min or less is nearly indistinguishable (Edwards et al., 1986). Despite the 5-fold difference, early Tf incorporation and steady state Tf levels in b/b reticulocytes are not diminished. This type of comparison has been repeated many times in our laboratory with Belgrade incorporation consistently 15–25% of normal, whereas Belgrade Tf incorporation is undiminished or even modestly elevated (1.0–2.0 × normal).

Hypothetically the decrease in iron uptake by Belgrade reticulocytes could be attributed to a mutation in either a serum or a cellular protein. A mutation that affects Tf function could result in a decrease in iron uptake by 1) a decreased affinity of iron–Tf for the TfR, 2) poor release of iron in the endocytic vesicle, or 3) an increased affinity of apoTf for the TfR, serum proteins other than Tf might also influence Tf’s ability to deliver iron, e.g. a serum component when mutated might inhibit Tf’s delivery of iron. Alternatively, a mutation that affects the iron delivery pathway in the reticulocyte could result in decreased iron uptake. An alteration in the TfR, in the processes of endocytosis or exocytosis or in a step between entrance and exit could influence the rate of iron delivery. The basis for the comparisons above was systematically analyzed by more specific comparisons below. Emphasis is always on whether the issue being tested can aid in the comparisons. Thus precise binding or rate constants are not the goals of this study; instead we wish to know if there are differences between b/b and normal reticulocytes, and if so, do the differences help account for the major decrease in iron uptake seen in reticulocytes from Belgrade rats.

Tf Functionally Normal—Although a defect exists in b/b reticulocytes incubated with normal Tf, one could argue that the defect is secondary to prolonged exposure to an abnormal Tf or to another altered serum protein. To see whether Belgrade Tf functions normally, we compared iron uptake into normal reticulocytes and heme from Belgrade versus normal serum. Fig. 1 shows that 59Fe incorporation into b/b reticulocytes is essentially the same from 30% saturated Belgrade versus +/b sera. Incorporation into heme is also similar for both sera. Rates of iron uptake into normal rat reticulocytes and heme were also indistinguishable for b/b versus +/b sera at both 50 and 80% iron saturation (not shown). Tf was also purified from Belgrade and normal rats and incubated with normal rat reticulocytes. No significant differences were found in the ability of b/b versus normal Tf to deliver iron into +/b cells or heme at 24 and 56% iron saturation (not shown).

tfR—The affinity of TfR on Belgrade versus normal reticulocytes for diferric Tf was measured by Scatchard analysis (Scatchard, 1949). Fig. 2 shows results for a typical experiment. Note that the plots for +/b and b/b are essentially indistinguishable. After least squares fittings, KD = 276 ± 79 nM for +/b and KD = 233 ± 59 nM for b/b (error limits = 1 S.D.). The two KD values are very similar. In addition to the data for surface TfR shown in Fig. 2, we made three more Scatchard analyses for surface TfR and three for total TfR with no detectable difference in KD between +/b and b/b in all repeats.

We were unable to obtain good estimates of the number of TfR per reticulocyte in these analyses. The problem is at least in part attributable to difficulties with the reproducibility and precision of the reticulocyte count, especially for b/b blood. The percentage of TfR on the surface could, however, be calculated after the same quantity of +/b cells was analyzed for both values. This value was 42% for the control; similar analyses yielded 50% for Belgrade cells. The same values were obtained in a second independent analysis; hence we will treat the distributions as slightly different. The relative numbers of TfR were estimated while determining the relative rates of endocytosis and exocytosis below; these ratios were combined with the surface percentages to calculate relative total numbers in the discussion.

\[
C = 1 - A - B \quad \text{(Eq. 3)}
\]

where \(A_0\) = the initial value for \(A\). This model did not fit the experimental data. The problem is that \(A\) was dissociating to \(C\) directly, i.e.

\[
A \leftrightarrow C \quad \text{(Reaction 2)}
\]

Solving the differential equations that describe this model proved intractable, so Equation 2 was altered to

\[
B = B_0 + D \cdot (k_1/(k_1 + k_2)) \cdot (A - A_0 \cdot e^{-k_1 t}) \quad \text{(Eq. 4)}
\]

where \(B_0\) = the initial value for \(B\); \(D\) = a proportionality constant that allows approximately for dissociation of \(A\) to \(C\) directly. This modification fits the experimental data reasonably well, because values for \(C\) stay in a narrow band.
Rate of Endocytosis—To see how the Belgrade mutation affects the endocytic process, we compared the rate of endocytosis of $^{125}$I-Tf in Belgrade reticulocytes to the rate in normal reticulocytes. Fig. 3A presents typical results. Accumulation of $^{131}$I counts/min/μg RNA internal to the cell, i.e. after Pronase treatment, is shown for the first 5 min of incubation. Uptake for b/b reticulocytes is slightly faster than for +/b cells. The probability they have the same rate constant is only 0.02 so the difference is significant; this significance is due to the very low deviation from least squares fitted values in this data set. The inset of Fig. 3A presents the same data transformed to a semilog format. This plot reveals b/b uptake to be 1.43-fold faster. Applying MINSQ to the main graphs yields these rate constants: b/b = 0.22 ± 0.01 and +/b = 0.15 ± 0.01 (estimate ± S.D.) to yield a ratio of 1.43 again.

The experiment shown in Fig. 3A was repeated four more times (not shown). The mean ratio of exponential rate constants was 1.5 ± 0.4 (b/b : +/b; range = 1.0–2.1); hence Belgrade endocytosis is unimpaired, probably increased. The maximum amount of Tf internalized, A, was also estimated with a mean ratio of 1.8 ± 0.9 (b/b : +/b; range = 0.9–3.3). This ratio confirms that Belgrade Tf incorporation is diminished and probably even increased.

Iron incorporation (Fig. 3B) was monitored concurrently to $^{125}$I-Tf incorporation. Linear incorporation for the +/b control confirms that $^{59}$Fe-$^{125}$I-Tf was unaffected by the labeling procedures. Iron incorporation by b/b cells was indistinguishable from control over the first 3 min, but detectably lower than normal at 5.3 min. This comparison was repeated four more times; during the first 3 min b/b incorporation was similar to normal (three comparisons) or slightly higher (two) but deviated to a lower rate by 5 min in all cases. Thus initial b/b iron entry is undiminished compared with normal but a defect becomes apparent within 5 min.

Rate of Exocytosis—We compared the rates of exocytosis in Belgrade versus normal reticulocytes to see how the Belgrade mutation affects this process. Reticulocytes were preloaded 30 min with $^{59}$Fe-$^{125}$I-Tf, stripped of surface TfR with Pronase, and then incubated in fresh medium with unlabeled Tf. Fig. 4A shows the loss of $^{125}$I-label from cells during the first 5 min of reincubation. The inset plots the same data in semilog form, as an internal $^{125}$I counts/min/μg RNA at a given time divided by the initial $^{125}$I counts/min/μg RNA (at time = 0). Belgrade reticulocytes have a 2.7-fold faster rate of exocytosis than normal. Applying MINSQ to the main graphs yields these rate constants: b/b = 0.31 ± 0.04 and +/b = 0.11 ± 0.01 (estimate ± S.D.) also yielding a ratio of 2.7. The experiment in Fig. 4A was repeated three more times (not shown). For each experiment the rate of exocytosis was faster for b/b reticulocytes averaging 2.2 ± 0.7-fold higher (estimate ± S.D., range 1.3–2.7). Three of four times the difference was significant (the fourth approaches significance); hence b/b Tf exit is faster than normal. The analyses also estimated the initial amount of Tf internalized, A, and the mean ratio was 1.8 ± 1.0 (range 0.9–3.3). Experiments were performed at the same time as those on endocytosis; thus the ratios for A are not independent of those for Aex. Agreement between estimates was good, again suggesting that Belgrade reticulocytes internalize somewhat more Tf than normal. Therefore the number of internal TfR in b/b reticulocytes is about 1.8 x than in normal reticulocytes. This ratio is based on an RNA denominator not per cell basis.

The extent to which $^{59}$Fe label was lost was also monitored (Fig. 4B). After 30 min of uptake prior to reincubation, essentially all of the iron was retained by the +/b control. Over 75% of the counts could be recovered from the cytosolic heme fraction (not shown), whereas about 18% were stromal and about 5% cytosolic non-heme. With heme being the main sink for iron in the reticulocyte, one would not expect any heme iron to be available for return to the medium during the chase period. In contrast, the Belgrade cells began the reincubation with fewer $^{59}$Fe counts/min/μg RNA present; this difference is a consequence of the decreased incorporation of
iron that is a hallmark of this mutant. Moreover, $b/b$ reticulocytes lost a portion of the $^{59}$Fe label under the same chase conditions. This loss could be fitted to the same exponential decay used to fit Belgrade data in A except that the initial value ($A_0$) of 42 declined to 29 instead of 0. Therefore about 30% of iron previously incorporated was exocytosed. Iron is not likely to be lost from the cytosolic heme fraction (≈60% of total incorporation, not shown) nor from cytosolic non-heme (~5%); but it could be derived from stromal iron (~35%). Hence twice as much of the iron label (35 versus 18%) is in the fraction from Belgrade reticulocytes that is most likely to be available for exocytosis. The analysis in B was repeated three more times; on each occasion the normal control exhibited no detectable decline in iron from about a 4 × higher level than Belgrade reticulocytes. Belgrade cells lost one-fourth to one-third of their label at an exponential rate, however, like that for loss of $^{125}$I-Tf.

Analysis of a Single Tf Cycle—Treating the differences for rates of endocytosis and exocytosis as real, we asked how these kinetic differences could be related to the 5-fold decrease in rate of Belgrade iron incorporation. One possibility was that the overall rate of Tf cycling accelerated in $b/b$ reticulocytes so that Tf remained in vesicles for too short an interval to permit delivery of its ligand iron. This possibility predicted a more rapid Tf cycle for Belgrade versus normal reticulocytes. A second possibility was that accelerated entry and exit of Tf were compensating for a block during an intracellular step. If this block slowed Tf cycle kinetics between endocytosis and exocytosis, then $b/b$ cycling would be similar to normal or even slower. If the block did not affect cycle kinetics, then $b/b$ cycling would be faster than normal. Fig. 5 tests these predictions. Before analyzing these data for the issue of comparative cycle times, we calculated expected results using a simple model for a Tf cycle ($A \rightarrow B \rightarrow C$, where $A$ = surface-bound Tf, $B$ = internal Tf, and $C$ = Tf in the medium; see Footnote 2 at end of “Materials and Methods”). For simplicity, this model omits the two kinetic steps between entry and exit. Because $+/?+$ and $b/b$ expectations were readily distinguishable, we proceeded to examine a single cycle of Tf movement for Belgrade versus normal reticulocytes. External TfR were loaded for 60 min with $^{59}$Fe-$^{125}$I-Tf at 0 °C; then cells were washed, placed into prewarmed medium at 37 °C, and reincubated for the times indicated in Fig. 5. $^{125}$I counts/min were determined for the medium, the cell surface and internal to the cell and were expressed as a fraction of the total $^{125}$I counts/min (1.00). The fraction of counts found in the medium in A was surprisingly large presumably because more than half of $^{125}$I-Tf comes off the TfR as soon as the cells are in fresh medium. We therefore altered the model (see Footnote 2) to allow for this phenomenon. Data in Fig. 5A were fitted by the modified model:

A cell surface $^{125}$I-Tf decreases with time in Fig. 5A as expected. Internal $^{125}$I-$^{125}$I decreases with time for the first 2 min as Tf and TfR are endocytosed. At later times (after 4 min) internal $^{125}$I-Tf decreases due to exocytosis. At 8 min the amount of internal $^{125}$I is less than at 0 min. This time dependence correlates nicely with $^{125}$I in the medium, one reflecting the other after taking surface Tf into account. All three curves are indistinguishable when Belgrade reticulocytes are compared with normal.

Absence of the expected difference could be explained by the existence of one or more kinetic steps in addition to endocytosis and exocytosis with at least one of the additional steps lengthened in Belgrade cells to compensate for more rapid uptake and release of Tf unless experimental scatter obscures faster cycling for $b/b$ reticulocytes. To explore this possibility we used the program MINSQ to calculate rate constants and their S.D. values as follows: $k_1$, the rate of internalization, i.e. surface to internal and $k_2$, the rate of externalization, i.e. internal to medium. If scatter were the dominant influence, we expected the rate constants to be of the same order as those found previously for endocytosis and exocytosis but the S.D. values to be much larger. If another kinetic process occurs, we expected one or both rate constants to be altered with the change reflecting MINSQ’s effort to factor the additional process into the calculation of the rate constant. For $+/?$ reticulocytes, $k_1$ was estimated as 0.24 ± 0.05 and $k_2$ as 1.07 ± 0.38. For Belgrade reticulocytes, $k_1$ was estimated as 0.19 ± 0.03 and $k_2$ as 0.93 ± 0.27. MINSQ reported that deviations were essentially normally distributed, so we tested significance of differences between control and Belgrade by a t test. The p values were 0.40 and 0.80, respectively, indicating that $+/?$ and $b/b$ are indistinguishable as can be seen in Fig. 5A. The values for $k_1$ are very similar to those obtained by direct measurement, but those for $k_2$ are considerably larger than the values for Fig. 4A. This observation and the fact that S.D. values exhibit larger increases for the $k_2$ values provide support for postulating the existence of at least one more kinetic process that is slower than normal in Belgrade Tf cycling.

The experiment shown in Fig. 5A was repeated six times. Although estimates of $k_1$ and $k_2$ varied considerably from one determination to another, $+/?$ and $b/b$ estimates within each determination did not differ significantly. Moreover, estimates for $k_1$ were within or close to the range observed for endocytosis; but those for $k_2$ were usually larger than the range observed for exocytosis. Hence we postulate that the
rate of a process between entry and exit into Belgrade reticulocytes is slower than normal. Remarkably, this difference almost exactly compensates for more rapid Belgrade endocytosis and exocytosis to yield indistinguishable Tf cycling times for b/b versus +/? cells.

Fig. 5B presents the data for iron distribution during the experiment for which A presents Tf distribution. To make comparison easier, the same model has been used to draw theoretical Tf curves in both parts, but lines are dotted for internal and medium in B. Note that surface iron fits the model equally well in both figures and that +/? and b/b data are again essentially the same. Both internal and medium iron do not fit the model, however, so additional curves (solid lines) were fitted visually to the data of Fig. 5B. These curves reveal that iron in the control unlike Tf continues to increase internally and decrease in the medium during the period from 2 to 5 min when endocytosis begins to dominate the Tf data. (Internal $^{55}$I-Tf peaks at 2 min for +/? versus about half the peak value at 5 min.) The behavior of +/? iron is in $B$ consistent with the argument that the iron is moving from endosomes through the cytosol to form heme in mitochondria and that this product (especially as hemoglobin heme) is unlikely to be released to the medium. After 8 min, one can see loss of some iron from the internal fraction to the medium in control reticulocytes. In contrast, b/b internal iron peaks at 3 min just after internal Tf (between 2 and 3 min; Fig. 5A) then decreases almost tracking the decrease for Tf but to only about half the peak level when Tf is down to about a fifth. The behavior of b/b iron suggests that release from Tf is inadequate or that iron relines to Tf after release because most $^{59}$Fe fails to transit the path of iron metabolism distal to endosomal dissociation of ferric Tf. Some iron that fails to move into heme in Belgrade cells must be returning to the medium.

Iron and Tf Released into the Medium—The observations in Fig. 5 draw attention to another aspect of endocytosis—the extent to which Tf and iron are released into the medium. Using a single cycle experiment to approach the issue is not appropriate, however, because the large fraction of surface Tf that is released directly to the medium obscures the release of iron and Tf that have undergone endocytosis and endosomal processing. We therefore used the exocytosis procedure to examine iron and Tf in the medium as $^{59}$Fe and $^{125}$I counts normalizing data to a ratio of 2.0 for diferric Tf. Pronase treatment removes Tf bound to surface Tf in this procedure before release of counts to the medium. Counts in the medium were determined instead of recovering Tf (e.g. after antibody precipitation), because there is no a priori assurance that all of the iron not retained within Belgrade reticulocytes is released associated with Tf. Fig. 6 combines data from five experiments. At times from 2 to 30 min after initiation of endocytosis both control and Belgrade cells release iron and Tf in approximately constant ratios of 0.9 and 1.7, respectively. Compared with the reference (input) ratio of 2.0, these values reveal that +/? and b/b cells, respectively, remove about 55 and 15% of input iron. This comparison accounts reasonably well for Belgrade reticulocytes incorporating $^{59}$Fe at about 20% the rate of control cells (after the first 3 min of incubation); 15/55 indicates the relative efficiency of b/b versus +/? iron utilization, about 27%.

**DISCUSSION**

We have eliminated several possible causes of the Belgrade defect within the Tf cycle by systematic comparisons, namely, Tf, the TfR with respect to affinity for iron · Tf and TfR number, and major alterations in rates of endocytosis, exocytosis, or cycling. Modestly increased incorporation of Tf during iron uptake is apparently due to a similar increase in TfR number (from estimates of $A_{\text{max}}$ and $A_I$). Comparisons of behavior of Tf-bound iron and iron kinetics and cycling reveal that iron uptake by Belgrade reticulocytes is initially similar to uptake by controls (Fig. 3B). Iron retention, however, is defective in b/b cells (Fig. 4B, 5B, and 6). A detectable fraction of $^{59}$Fe returns to the medium during single cycle (Fig. 5B) or exocytosis (Figs. 4B and 6) experiments with Belgrade cells even though the counts do first enter the cells. As a result, Belgrade reticulocytes utilize much less Tf-bound iron for heme synthesis. Below we consider the systematic comparison, how well conclusions from individual comparisons agree with one another, how to resolve apparent inconsistencies, and interpretation of the whole set of comparisons.

**The Tf Molecule and Other Serum Components—Belgrade Tf is functionally normal.** No significant differences in iron delivery were seen when Belgrade serum was compared with normal serum at several iron saturations (Fig. 1 and text). Young et al. (1984) showed previously that rabbit TfR bound apo-, monoferric, and diferric Tf differently; hence it was critical to look at a range of iron saturations. Serum was examined to determine whether another protein was either aiding Tf function in control serum or interfering with it in the mutant. Purified Tf's from Belgrade and normal rats also showed no differences in iron delivery at differing saturations with iron. Thus, the anemia seen in this rodent mutant is not the result of a functionally different Tf. Farcich and Morgan (1992) reached a similar conclusion after studying the ability of purified Belgrade and Wistar Tf to donate iron to reticulocytes and to compete with each other during TfR binding.

**TfR—No obvious defect is seen in the Belgrade TfR with respect to affinity for iron · Tf (Fig. 2).** Bowen and Morgan (1987) were also unable to distinguish Belgrade versus control TfR, although $K_d$ values calculated from their data are about half of ours. In Belgrade reticulocytes internal TfR are elevated in number. The degree to which they are elevated (1.8 X) combined with the estimates that 42 or 50% of the TfR are on the surface, respectively, for +/? or b/b, permit one to calculate the relative number of total TfR. Thus Belgrade cells have the same number of TfR on the surface as they do internally, while normal reticulocytes have 42/58 = 0.7 X as many TfR on the surface. Thus if i = the number of internal TfR for +/? reticulocytes, total TfR for control as a fraction of Belgrade = (i+?)/(b/b) = 1.7i/(1.8i + 1.8i) = 1.2. This ratio is also supported by fluorescent microscopic observations (not shown) after reacting normal versus Belgrade reticulocytes with murine monoclonal antibody directed against rat
TfR then with fluorescent tagged goat anti-mouse immunoglobulin G.

A mutation in the TfR could still be the cause of the Belgrade defect. Recently, Sipe and Murphy (1991) and Bali et al. (1991) have shown that the TfR participates actively in facilitating the release of iron from Tf. Thus as pH decreases from neutrality to as low as pH 5.6, our Scatchard analyses were only at pH = 7.4. The effect of pH on the release of iron from TfR then with fluorescent tagged goat anti-mouse immuno-taken UP previously in a 10-min preincubation during a sub-sequent 20-min chase, whereas control cells exhibited no loss. This type of loss, associated with Tf exocytosis, must become noticeable within 5 min after uptake, accounting for the lag seen within 5 min after the start of iron endocytosis for Belgrade cells in Fig. 3B. Possibly such shorter preincubation would reveal a small loss from normal reticulocytes associated with exocytosis and consistent with their behavior in single cycle analysis (Fig. 5 and discussed below).

**Single Cycle Analysis**—Single cycle experiments (Fig. 5) did not show any difference between Belgrade and normal reticulocytes with respect to rate of endocytosis and failed to detect the difference in exocytosis rates observed previously (Fig. 4). We postulate that Belgrade Tf cycling slows during an intracellular step, balancing the effect of slightly more rapid entry and 2 × more rapid exit.

Exocytosis rate constants are larger in single cycle analysis than in direct estimates. This paradox is probably due to Pronase treatment of reticulocytes after preincubation during exocytosis measurements. Removal of external TfR and other surface damage could slow exocytosis. It is difficult, however, to explain why Pronase slows Belgrade exocytosis less than normal.

The existence of a kinetic step between entry of ligand into cells and exit of the TfR is essential if any intracellular processing of the ligand-TfR complex occurs. Ciechanover et al. (1983) postulated such an intermediate kinetic step for the TfR, involving dissociation of iron from Tf and iron storage, in their analysis of Tf cycling in hepatoma cells. To obtain a satisfactory fit to their data, they broke the process equivalent to our rate $k_1$ into two processes with distinct rate constants, $k_1$ and $k_2$, with the former for the intracellular step(s) and the latter, for exocytosis. Nunez and Glass (1983) pulse-labeled rabbit reticulocytes with $^{59}Fe^{125}I$-Tf, then chased with unlabeled diferric Tf to reveal that release of iron from Tf is an extremely fast event after initial binding and internalization. If normal rat reticulocytes are similar, we might have difficulty discerning the kinetic hallmarks of this process, yet still see the effects of slowing it in Belgrade reticulocytes as an unaltered Belgrade cycle time.

Cycling data for iron (Fig. 5) show that a small portion (~10%) of iron that enters normal cells subsequently returns to the medium, but a much larger portion (~50%) of Belgrade iron returns to the medium. The time courses for internal iron and iron in the medium follow those for Tf initially, but lag slightly behind for the internal peak and the return to the medium reflecting additional kinetic process(es) affecting iron distribution, such as endosomal acidification and the exit of iron from vesicles en route to mitochondria. These processes first affect the results for iron differentially relative to those for Tf and begin to distinguish $+/?$ from $b/b$ in the period after 2–3 min of incubation. This set of relationships accounts for the data on endocytosis (Fig. 3B) where Belgrade iron incorporation begins to lag behind normal between 3 and 5 min after the incubation has started.

**Iron and Tf Returned to the Medium**—Because rat reticulocytes release a large portion of surface bound $^{59}Fe^{125}I$-Tf directly to the medium without endocytosis (Fig. 5, viewed in light of models in Footnote 2), we determined the extent to which iron and Tf are returned to the medium after endocy-tosis and Pronase removal of surface bound Tf and TfR (Fig. 6). Although data are expressed as iron/Tf ratio, one should keep in mind that the medium was counted, not a recovered Tf fraction. This approach is used because the overall release of transferrin and iron is the issue not just how much iron was associated with Tf. Belgrade reticulocytes released an essentially constant ratio of 1.7 over the period of 2–30 min, whereas control cells released a ratio of 0.9. Taking 2.0 as an input ratio, Belgrade cells utilize iron at $(2.0 - 1.7)/2.0 -$
0.9) = 0.3/1.1 ≈ 27% the efficiency of normal. This efficiency is within experimental error of the 20% efficiency directly measured for rates of iron incorporation into cells or heme. Edwards et al. (1986) used a different experimental design to obtain very similar data. They examined Tf in the medium after Belgrade versus control cells released doubly labeled Tf to the medium during a 60-min incubation. Control reticulocytes removed 60% of the iron, but Belgrade cells only removed 16%. Remarkably 16/60 ≈ 27%. Thus Belgrade reticulocytes release iron at a rate reflecting defective utilization.

That only 55% of input iron is removed from dipheric Tf by normal rat reticulocytes was an unexpected finding in view of the appearance that a larger fraction is removed in single cycle experiments (Fig. 5) and a general sense that iron is removed from dipheric Tf nearly totally by erythroid cells. The discrepancy between Figs. 6 and 5 for the extent of iron release by normal cells is accounted for singly or in combination by the following possibilities. 1) Exocytosis measurements are made on Pronase-treated cells that release only internal iron and Tf. Single cycle experiments, however, involve release of surface bound iron and Tf to the medium in addition to iron and Tf that has been taken up by the cells. The amount of this surface release during single cycle experiments obscures the actual proportion of iron returned to the medium from within normal cells. 2) Although surface iron is permitted entrance into cells from the initiation of a single cycle experiment, entry occurs over the length of the incubation not just at its beginning; integrating iron utilization over this period leads to the appearance of a more efficient utilization. 3) Dipheric Tf released from the surface of cells to the medium during a single cycle experiment rebinds to the cells during the length of the incubation also contributing to the appearance of a more efficient utilization. A similar set of arguments accounts for the discrepancy for data on Belgrade cells after acknowledging that they released more iron to the medium and retained less intracellularly in both figures.

Edwards et al. (1986) is not the only study that previously reported the extent of iron removal from Tf in normal erythroid cells; Hradilek and Neuwirt (1987) have reported data on iron unloading from Tf in induced and uninduced Friend erythroleukemia cells. There is clearly little release of surface bound Tf directly to the medium without cycling internally in this system. We calculate from their data that induced cells, an erythroid model, remove about 55% of input iron from dipheric Tf, whereas uninduced cells remove about 40%. Hence our finding of 55% for normal rat reticulocytes is quite similar to their data on a murine erythroid model.

The Belgrade Defect—We have systematically compared Belgrade versus normal rat reticulocytes for components and steps of the Tf cycle to look for differences that would account for Belgrade iron utilization at only 20% the efficiency of normal. The data exclude differences in Tf and in the affinity of TfR for Tf under the conditions of Scatchard analyses. Modest differences in TfR numbers and distribution plus rates of Tf entry and exit are consistent with one another but do not lead to altered single cycle kinetics much less readily accounting for a decrease in iron uptake to only 20% of normal. Iron is simply not leaving Tf after essentially unobstructed entry into the Belgrade endosome. Tf is departing the endosome for the cell surface where it is released accompanied by about twice as much unutilized iron in the case of b/b reticulocytes than normal. These observations are indicative of defective iron release inside Belgrade endocytic vesicles or the absence of a system that normally aids iron in transit from endosomes to mitochondria. Such a failure leads to iron remaining or reassociating with Tf and being released to the medium. Using fluorescently tagged Tf, we (Garrick et al. 1990c, 1990d) have recently demonstrated defective endosomal acidification in intact Belgrade reticulocytes. An elevated endosomal pH would account for all of the differences and similarities seen for Belgrade versus control rat reticulocytes in the present study.

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