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Release of Iron from the Two Iron-Binding Sites of Transferrin by Cultured Human Cells: Modulation by Methylamine†

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ABSTRACT: We have investigated the effect of increasing concentrations of methylamine (5, 10, and 25 mM) on the removal of iron from the two iron-binding sites of transferrin during endocytosis by human erythroleukemia (K562) cells. The molecular forms of transferrin released from the cells were analyzed by polyacrylamide gel electrophoresis in 6 M urea. Endocytosis of diferric transferrin was efficient since <10% of surface-bound protein escaped endocytosis and was released in the diferric form. Although transferrin exocytosed from control cells had been depleted of 80% of its iron and contained 65-70% apotransferrin, iron-bearing species were also released (15% C-terminal monoferric; 10% N-terminal; 10% diferric). The ratio of the two monoferric species (C/N) was 1.32 ± 0.12 (mean ± SD; $n = 4$), suggesting that iron in the N-terminal site was more accessible to cells. In the presence of methylamine there was a concentration-dependent increase in the proportion of diferric transferrin release (>80% at 25 mM) and a concomitant decrease in apotransferrin. Small amounts of the iron-depleted species, especially apotransferrin, appeared before diferric transferrin, suggesting that these were preferentially released from the cells. The discrepancy between the proportions of the monoferric transferrin species noted with control cells was enhanced at all concentrations of methylamine, most markedly at 10 mM when the C/N ratio was 2.4. The N-terminal site of transferrin loses its iron at a higher pH than the C-terminal site, and so by progressively perturbing the pH of the endocytic vesicle we have increased the difference between the two sites observed with control cells. The observation that the in vitro site differences apply during interaction of transferrin with cells suggests that protonation of the sites is of prime importance in iron release.

The plasma iron-transport glycoprotein transferrin consists of a single polypeptide chain (M_r 80000) bearing two specific binding sites that can each bind an atom of iron with high affinity (Morgan, 1974). Because the binding of iron to transferrin displaces protons from the protein, the equilibrium can be perturbed by lowering the pH, and this method has been widely used to remove iron from transferrin during studies of the protein-metal interaction (Lestas, 1976; Princiotto & Zapolski, 1975; Aisen et al., 1978). Similarly, reductive removal of iron from transferrin can be achieved since Fe(II) is only weakly bound (Gaber & Aisen, 1970), but this only occurs rapidly if an agent to displace the coanion bicarbonate from the protein-iron complex is present (Ankel & Petering, 1980). Thus two nondestructive methods are available to overcome the kinetic barrier to the release of iron from the transferrin, and it seems likely that cells make use of one or both of these methods to remove the iron. Some evidence suggests that transferrin iron becomes available to chelators of Fe(II) within the cell and therefore passes through a stage at which it is reduced (Morgan, 1971; Thorstensen & Romslo, 1984), and it is now realized that the protein encounters an environment at low pH during its transit through the cell. However, a more definitive conclusion on the relative importance for cellular acquisition of iron of reduction and protonation is not yet possible.

The initial event in cellular iron uptake involves the binding of iron-rich transferrin to specific cell-surface receptors that are found on erythroid cells (Jandl & Katz, 1963; Iacopetta & Morgan, 1983), normal nonerythroid cells (Young & Aisen, 1980; Galbraith et al., 1980; Hamilton et al., 1984), and a wide variety of cultured cell lines (Seligman, 1983). Some workers have interpreted data on the binding and release of transferrin from cells (Woodworth et al., 1982) and on the accessibility of iron to chelating agents during uptake from transferrin (Nunez et al., 1983; Nunez & Glass, 1983) as indicating that

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iron release from transferrin takes place at the plasma membrane. An alternative view is that the transferrin–receptor complex is endocytosed and subsequently exposed to an acidic environment (Iacopetta & Morgan, 1983; Klausner et al., 1983a; Ciechanover et al., 1983). Acidification of endocytic vesicles could be achieved by fusion with lysosomes (Octave et al., 1981, 1982; Karin & Mintz, 1981), but more recent biochemical and morphological studies of vesicles containing transferrin indicate that the protein is processed in a distinct prelysosomal compartment (van Renswoude et al., 1982; Lamb et al., 1983; Dickson et al., 1983; Harding et al., 1983).

A number of physicochemical studies of transferrin have shown the two sites are nonequivalent in their iron-binding properties (Evans & Holbrook, 1975; Evans & Williams, 1978; Donovan, 1977). The observation that the two sites also have different susceptibilities to proton attack in vitro (Princiotto & Zapolski, 1975; Lestas, 1976) is of particular importance because of the suggestion that transferrin is exposed to an acidic environment in the cell. By using a combination of spectroscopic analysis and polyacrylamide gel electrophoresis in 6 M urea (Makey & Seal, 1976) to separate the monoferric species, it has been possible to identify the iron-binding site that is particularly susceptible to protonation (Aisen et al., 1978).

In the present study we have used the latter technique to investigate the release of iron from the two iron-binding sites of diferric transferrin during its interaction with K562 cells, a cultured human leukemic cell line, in which the endocytosis of transferrin has been previously documented (Klausner et al., 1983a,b; Enns et al., 1983). Analysis of the transferrin species released from the cells showed that a significant proportion consisted of the monferric transferrins. When increasing concentrations of the weak base methylamine were used to induce a progressive perturbation of the pH of intracellular vesicles (Poole & Okhuma, 1981), we amplified the differences, noted previously in vitro (Princiotto & Zapolski, 1975; Lestas, 1976), in the susceptibility to protonation between the two iron-binding sites of transferrin.

Materials and Methods

Materials. MEM,[1] RPMI 1640, and fetal calf serum were obtained from Gibco Europe, Paisley, Scotland, U.K., and all plastics for tissue culture were from Sterilin, Teddington, Middlesex, U.K. BSA (fraction V), methylamine, and DTPA were from Sigma London, Poole, Dorset, U.K., and Pronase B (Calbiochem) was from CP Laboratories, Bishops Stortford, Herts, U.K. Chelex 100 was bought from Bio-Rad Laboratories, Bromley, Kent, U.K., and desferrioxamine mesylate was from CIBA Laboratories, Horsham, Sussex, U.K.

Transferrin Preparation and Radioisotopic Labeling. The methods used to purify transferrin from pooled human plasma and to label it with ^{125}I (Amersham International, Bucks, U.K.) by solid-phase lactoperoxidase treatment have been described in full elsewhere (Bomford et al., 1983). Specific activities were in the range 800–1400 counts per minute (cpm)/ng of transferrin.

Cell Culture. K562 cells were obtained from the Imperial Cancer Research Fund, London, U.K., and grown in RPMI 1640 supplemented with 25 mM Hepes, 10% (v/v) fetal calf serum, and antibiotics (penicillin, 50 units/mL; streptomycin, 50 μg/mL). Cells were cultured at 37 °C in 75-cm^2 tissue culture flasks at densities of 5×10^5 to 1×10^6 cells/mL. Cells were maintained in log phase by dividing 1 to 2 with fresh medium every 48 h. Experiments were performed on cells that had been fed 24 h previously. Before use all cells were incubated 3 times at 37 °C for 15 min in MEM and 1% BSA to free them of endogenous transferrin (Hemmaplardh & Morgan, 1974). Cell viability was >98% as assessed by trypan blue exclusion.

Release of ^{125}I-Labeled Transferrin for Electrophoresis. Washed cells [(40–80) $\times 10^6$ cells/mL] were incubated in MEM and 1% BSA at 37 °C for 15 min with 310 nM ^{125}I-labeled transferrin to saturate the full complement of receptors or at 4 °C for 1 h to occupy surface receptors only. In some experiments 5, 10, or 25 mM methylamine was present. The cells were then washed 3 times in medium, containing methylamine if indicated. Subsequent steps in the experiments were carried out by using acid-washed apparatus and iron-depleted medium. The latter was prepared by passing MEM and 1% BSA containing 25 mM Hepes over Chelex 100 equilibrated in 25 mM Hepes, pH 7.5. The concentrations of Ca^{2+} and Mg^{2+} were restored to 1.8 and 0.8 mM, respectively, after this treatment. To scavenge remaining free iron, shown to be present in commercial media (Young, 1982; Brock, 1981), this medium was then made 5 mM in DTPA or 100 μM in desferrioxamine mesylate or 6.25 μM in human apotransferrin and was incubated for 1 h at 37 °C and for 2 h at 4 °C. These concentrations of chelators were found to be the minimum required to maintain ^{125}I-labeled apotransferrin free of iron in a cell-free incubation when the protein was analyzed by polyacrylamide gel electrophoresis in urea. Under these conditions ^{125}I-labeled diferric transferrin retained >96% of its iron. Just before use the media were made 310 nM with unlabeled diferric human transferrin.

Cold iron-free medium (0.3–1 mL) was added to (30–80) $\times 10^6$ cells on ice, and the suspension was gassed with 5% CO_2/air. The cells were then rapidly warmed to 37 °C and after an interval centrifuged for 2 min at 800g(av) at 4 °C. This cycle was repeated with fresh prewarmed medium, and the harvested supernatants were kept on ice and analyzed by electrophoresis and by γ counting. Samples of the cells were removed during successive incubations and washed once in cold PBS, and the accessibility of the ^{125}I label to Pronase B (Klausner et al., 1983a) and cell viability also were determined. Cell viability was always >98%.

Analysis of the Iron Status of Released ^{125}I-Labeled Transferrin. The cell supernatants were analyzed by electrophoresis in 6% polyacrylamide rod gels (5.5 × 100 mm) containing 6 M urea as previously described (Young, 1982; Groen et al., 1982). Huebers et al. (1984) have pointed out that this technique can lead to iron contamination of the transferrin in the sample and exchange of iron between the two iron-binding sites unless precautions are taken. However, we have previously shown that in our hands the four molecular species of transferrin, isolated by preparative urea–polyacrylamide gel electrophoresis, remain unaltered, neither gaining nor losing iron during an incubation in culture medium containing iron chelators, followed by analytical urea electrophoresis (Young et al., 1984). After electrophoresis the gels were sliced with a lateral gel slicer for γ counting. The amounts of the different transferrin species on the gels were obtained by summing the counts in each peak. Radioactivity in the nodal slice between the two monoferric species was distributed equally between the two species. All of the radioactivity on the gel was included in the peaks.

[1] Abbreviations: MEM, minimum essential medium; BSA, bovine serum albumin; DTPA, diethylenetriaminepentaacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline (Dulbecco).

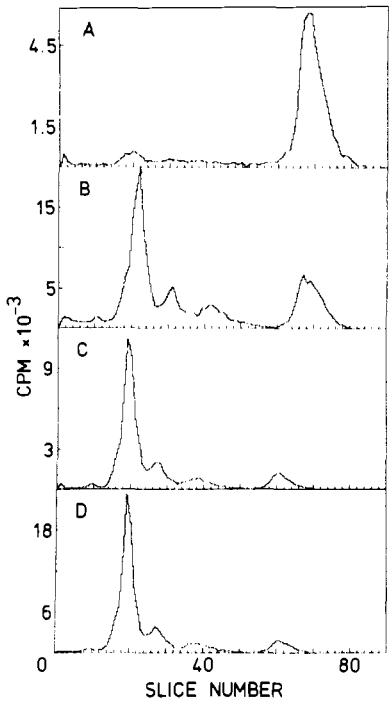

FIGURE 1: Electrophoretic analysis of the molecular forms of transferrin released from K562 cells. Cells (40×10^6) were incubated with 310 nM ^{125}I-labeled diferric transferrin at 4 °C for 1 h to coat the surface receptors or at 37 °C for 15 min to occupy the full complement of receptors. After the cells were washed at 4 °C, they were reincubated in 0.3–1 mL of medium containing 6.25 μM unlabeled apotransferrin and 310 nM diferric transferrin. The medium had been treated as described under Materials and Methods to remove free iron. The cells were warmed to 37 °C for 1 min, the medium was recovered by centrifugation, and fresh, prewarmed medium was added to the cells for a further 4 min. Aliquots (25–50 μL) of the media were analyzed by polyacrylamide gel electrophoresis in 6 M urea. Slice 1 represents the origin of the gel in each case. Panels A and B show the material released after 1 and 5 min, respectively, by cells initially coated with transferrin at 4 °C. Panels C and D show that released at 1 and 5 min, respectively, by cells preincubated with transferrin at 37 °C.

^{125}I-Labeled Transferrin Endocytosis and Exocytosis. The location of ^{125}I-labeled transferrin during the endocytic cycle in the cells used in this study was examined by incubating cells at 4 °C with 310 nM ^{125}I-labeled diferric transferrin in MEM and 1% BSA for 1 h and washing 3 times at 4 °C in medium to remove unbound ligand. Cells were then resuspended in fresh medium containing 310 nM unlabeled transferrin at 4 °C and rapidly warmed to 37 °C. At intervals, cells were sampled into 0.5 mL of medium at 4 °C and recovered by centrifugation, and the accessibility of the cell-associated ligand to Pronase (100 μg/mL) was determined by incubating the cells for 20 min at 4 °C. The transferrin released from the cells and the radioactivity accessible and inaccessible to Pronase were determined by using an LKB Compugamma γ counter.

RESULTS

Identification of Molecular Species of Released Transferrin. Transferrin was analyzed after its release from cells that had been prepared in two ways. In the first experiment cells were coated with ^{125}I-labeled transferrin at 4 °C, washed to remove unbound ligand, and then warmed to 37 °C. As shown in Figure 1A, nearly all the transferrin released into the medium during 1 min at 37 °C was iron rich with 85% migrating as the diferric species. After 5 min (Figure 1B) the iron status of the released transferrin had changed, with 50% migrating as apotransferrin, 25% as the diferric form, and 15% and 11% as the C- and N-terminal monoferric species, respectively.

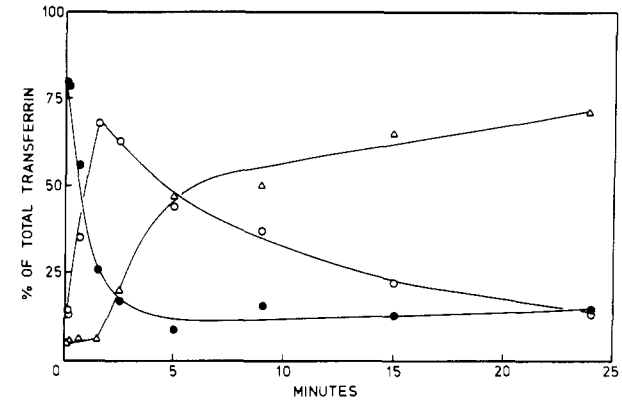

FIGURE 2: Endocytosis of surface-bound diferric transferrin by K562 cells. Cells (40×10^6) were incubated at 4 °C with 310 nM ^{125}I-labeled diferric transferrin for 1 h and washed 3 times at 4 °C to remove unbound ligand. They were then warmed to 37 °C in fresh MEM and 1% BSA containing 310 nM unlabeled diferric transferrin and sampled into cold medium. After recovery by centrifugation the cells were incubated at 4 °C for 20 min in MEM containing 100 μg/mL Pronase, and the cells were again recovered by centrifugation. Radioactivity released from the cells on warming to 37 °C (△) and that associated with the cells which was accessible (●) and resistant (○) to Pronase were measured by γ counting.

In the second experiment, in which the initial incubation with ^{125}I-labeled diferric transferrin was performed at 37 °C in order to occupy the full complement of receptors, the transferrin released by 1 min had been freed of most of its iron (Figure 1C). The predominant species was apotransferrin (65%) with 15% and 10%, respectively, of the C- and N-terminal monoferric transferrins. By 5 min (Figure 1D) the proportion of apotransferrin had risen to 70%, and there was little change in the proportions of the monoferric transferrins.

The diferric transferrin released from the cells that had been coated with transferrin at 4 °C (Figure 1A) represented only 6% of the total radioactivity bound. Much of this was released from the surface of the cells since at this stage 50% of the radioactivity could be removed from the cells by using Pronase. To examine in more detail the origin of the transferrin released under these conditions, cells were again coated with transferrin at 4 °C and then warmed to 37 °C to initiate endocytosis. The accessibility of the cell-associated transferrin was then investigated. Initially, a small proportion (~5%) of the cell-associated transferrin was lost into the medium (Figure 2), and since the majority of the protein was still accessible to Pronase and by inference was on the cell surface, this represented transferrin lost from the surface without endocytosis. Our electrophoretic analysis indicated that the majority of this was diferric transferrin (Figure 1A). After a minimum delay of 2 min there was an increase in transferrin appearing in the medium (Figure 2). By this time maximum endocytosis of the protein had occurred and internalized protein had begun to decline (Figure 2). This exocytosed transferrin corresponded to the iron-depleted protein we had shown to be released from the cells (Figure 1B).

Concentration- and Time-Dependent Effects of Methylamine on the Molecular Species of Released Transferrin. The rates of uptake (Figure 3) and steady-state binding (Table I) of transferrin by the cells were unaffected by methylamine. There was, however, a marked slowing of transferrin release from cells, and this effect was dependent on the concentration of methylamine used (Figure 4; Table I).

Control cells and cells preincubated with increasing concentrations of methylamine were loaded with ^{125}I-labeled diferric transferrin at 37 °C, and the released transferrin was

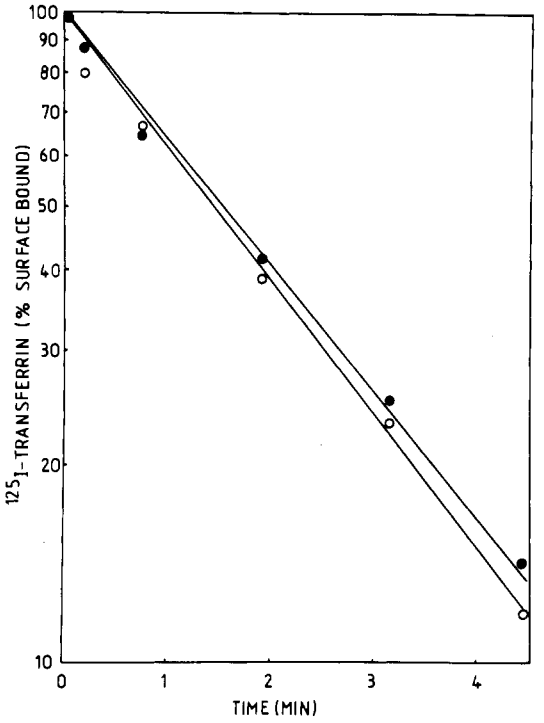

FIGURE 3: Effect of methylamine on the endocytosis of transferrin by K562 cells. Cells (40×10^6) were preincubated for 15 min at 37 °C in medium alone (○) or in medium with 25 mM methylamine (●) and then cooled to 4 °C. ^{125}I-Labeled diferric transferrin (310 nM) was added and the incubation continued for 1 h at 4 °C. The cells were then washed at 4 °C in medium with 25 mM methylamine if indicated, warmed to 37 °C in fresh medium and 1% BSA containing 310 nM unlabeled diferric transferrin with or without 25 mM methylamine, and sampled at intervals into cold medium. The accessibility of the cell-associated ^{125}I-labeled diferric transferrin to Pronase was determined as described in the legend to Figure 2. The rate constants for endocytosis by control (○) and methylamine-treated cells (●) were derived from semilogarithmic (ln) plots of the disappearance of transferrin from the cell surface with time.

Table I: Effect of Methylamine on Uptake and Release of Transferrin by K562 Cells[a]

conditions	rate const, endocytosis (min^{-1})	transferrin uptake (%)	rate const, exocytosis (min^{-1})	$t_{1/2}$, exocytosis (min)
control	0.45	100	0.1	6.9
methylamine, 5 mM		97	0.028	24.8
methylamine, 10 mM		95	0.019	36.5
methylamine, 25 mM	0.4	87	0.015	46.2

[a] Cells were preincubated for 15 min at 37 °C in medium and methylamine if indicated. ^{125}I-Labeled diferric transferrin (310 nM) was added, and at intervals samples of the cells were washed 3 times in PBS at 4 °C. Binding of transferrin at 40 min was corrected for nonspecific binding and expressed as a percentage of control binding (60 ng/10^6 cells). At 75 min the remaining cells were washed 3 times in cold medium containing the required concentration of methylamine and resuspended in medium containing methylamine and 310 nM unlabeled differic transferrin. The decrease in cell-associated ^{125}I-labeled transferrin was determined on warming the cells to 37 °C. The rate constants for release were derived from a semilogarithmic (ln) plot of cell-associated transferrin vs. time. Endocytosis rate constants were derived as described in Figure 3.

analyzed by electrophoresis. The proportions of each of the four species were determined after their release during a sequence of five 2-min incubations at 37 °C, replacing the medium, on each occasion, with fresh prewarmed medium. The majority of the transferrin released in the absence of methylamine was apotransferrin (Figure 5A). In the presence

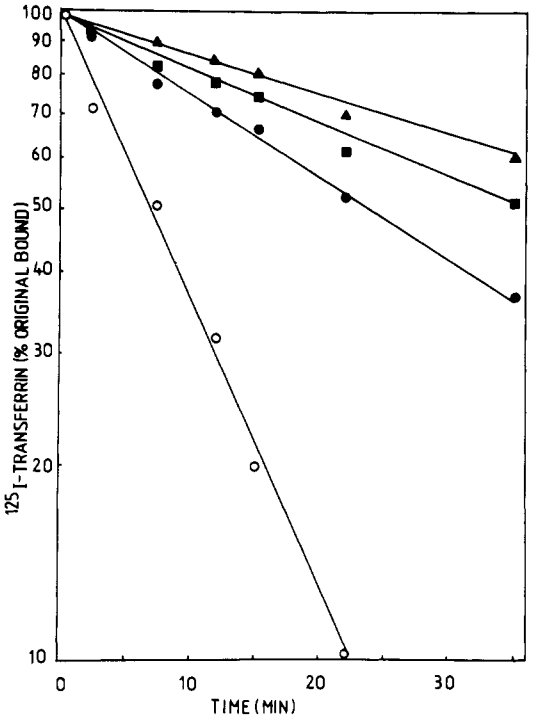

FIGURE 4: Effect of increasing concentrations of methylamine on the release of transferrin from K562 cells. Cells (40×10^6) were preincubated with the required concentration of methylamine or medium alone and then loaded with ^{125}I-labeled diferric transferrin at 37 °C as described in Table I. After the cells were washed at 4 °C in media containing the required concentration of methylamine, the decrease in cell-associated ^{125}I-labeled transferrin was determined on warming the cells to 37 °C in media containing 310 nM unlabeled diferric transferrin. Shown is the release from control cells (○) and cells incubated with 5 (●), 10 (■), and 25 mM (▲) methylamine.

of methylamine, and most markedly at 25 mM, diferric transferrin represented a major proportion of the protein released, and the proportion of apotransferrin became less with increasing methylamine concentration (Figure 5B–D). In the absence of methylamine, the proportions of the various transferrin species released did not change with time (Figure 5A). In contrast, in the presence of methylamine, during successive incubations, the proportion of diferric transferrin increased while those of the apotransferrin and monoferric transferrin species fell. With 25 mM methylamine, apotransferrin and the monoferric transferrins represented 60% of the protein released during the first reincubation (Figure 5D). However, only 10% of the total cell-associated transferrin was released during this incubation, and even at the end of the sequence of incubations 60% of the protein remained with the cells since the rate of release was much decreased (Table I; Figure 4) compared with that of the control cells, from which 93% of the transferrin was released. During the last three incubations 70–80% of the transferrin released was diferric transferrin (Figure 5D). Thus, although apotransferrin and the monomeric transferrins were predominant during the first incubation, the majority of transferrin release during the complete series of incubations with 25 mM methylamine was diferric transferrin.

With 5 and 10 mM methylamine the overall saturation with iron of the released transferrin was lower than with the higher concentration, with a greater proportion of the monoferric transferrin and apotransferrin present. As with 25 mM methylamine, and in contrast to the control incubations, the proportion of the iron-depleted species was high during the initial incubation and declined with time (Figure 5B,C). This indicates that the iron-depleted transferrin species, especially

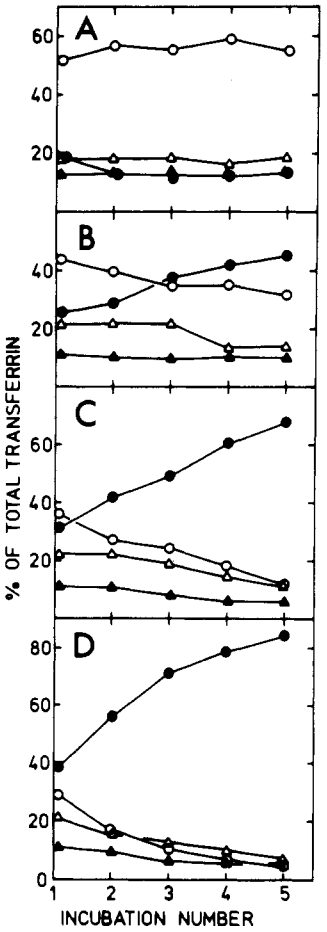

FIGURE 5: Effect of methylamine on the molecular forms of transferrin released by K562 cells. Cells (40×10^6) were preincubated for 15 min at 37 °C with medium alone (A) or with 5 (B), 10 (C), or 25 mM (D) methylamine before the addition of 310 nM ^{125}I-labeled diferric transferrin. After 15 min at 37 °C the cells were washed 3 times in cold medium containing methylamine if indicated. All media were treated as described under Materials and Methods to minimize free iron. An aliquot (0.3 mL) of prewarmed medium containing 6.25 μM unlabeled apotransferrin, 310 nM diferric transferrin, and methylamine if required was added. The medium was then recovered after 2 min by centrifugation at 4 °C, and fresh prewarmed medium was added. This cycle was repeated 5 times and 25–50 μL of each medium analyzed by electrophoresis in 6 M urea. The percentage of apotransferrin (○), C-terminal monoferric transferrin (△), N-terminal monoferric transferrin (▲), and diferric transferrin (●) released from the cells during each 2-min incubation is shown.

apotransferrin, were released preferentially at the beginning of the incubation.

Release of Monoferric Transferrins. The two monoferric transferrins accounted for between 25% and 30% of the transferrin released from control cells, suggesting that the removal of iron from the protein within the cells was not complete even in control cells. The proportions of the two species under these conditions varied little during the course of (Figure 5A) or between (Figures 1B,C and 5A) incubations. Iron removal from the two sites appeared to be nonequivalent since consistently more C-terminal than N-terminal monoferric transferrin was released. The ratio C/N from four independent experiments was 1.32 ± 0.12 (mean ± SD).

As shown in Figure 5B–D, compared with the control cells (Figure 5A), C-terminal monoferric transferrin was further increased relative to the N-terminal species in the presence of all concentrations of methylamine. This discrepancy between the two species was most marked in the presence of 10 mM methylamine, as illustrated by the analysis (Figure 6) of the medium from the third incubation, where C/N was 2.4.

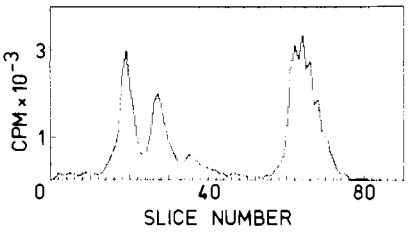

FIGURE 6: Electrophoretic analysis of the molecular forms of transferrin released from K562 cells treated with 10 mM methylamine. Shown is an analysis of the ^{125}I-labeled transferrin released from cells incubated as in Figure 5C (third sample). The ratio C/N of the monoferric transferrin was 2.4, compared with 1.5 in control cells.

The mean values for the five incubations shown in Figure 5 in the presence of methylamine were 1.9 (5 mM), 2.1 (10 mM), and 1.8 (25 mM).

Discussion

A comparison of the rate of iron uptake from transferrin with the mean length of an endocytic cycle in an erythroid cell (Iacopetta & Morgan, 1983) and nonerythroid cells (Ciechanover et al., 1983) suggests that apotransferrin should be released from cells. Our direct analysis of released transferrin confirmed that the majority (60–70%) was indeed apotransferrin (Figure 1C,D). This means that approximately 80% of the iron had been removed from the diferric transferrin by the cells. However, a substantial proportion of the transferrin was released as the iron-bearing species (25–30% monoferric and 10–15% diferric), and the proportion of these species remained constant throughout an incubation using control cells (Figure 5A). Since the transferrin added to the original incubation was 96% diferric transferrin, when analyzed by urea–polyacrylamide gel electrophoresis, and endocytosis of the surface-bound transferrin was essentially complete (94%; Figure 2), the monoferric transferrins could not have been generated at the cell surface. The source of the diferric transferrin is less clear. Some of it can be accounted for by the release from the cell surface of transferrin that had escaped endocytosis or was nonspecifically bound to the surface, and this amounted to approximately 6% (Figure 2). In addition, a small amount of diferric transferrin, which appeared during a series of incubations of control cells (Figure 5A), was probably exocytosed along with the monoferric species. The exocytosis of iron-bearing forms of transferrin suggests that iron removal from diferric transferrin may not be completely efficient. This may be a general feature of the uptake of transferrin by cells, as it has previously been shown that some monoferric transferrin accumulated during the incubation of diferric transferrin with rat reticulocytes and hepatocytes (Young, 1982) and rabbit reticulocytes (van Baarlen et al., 1980). We found, in agreement with a number of previous studies (Karin & Mintz, 1981; Morgan, 1981; Iacopetta & Morgan, 1983; Ciechanover et al., 1983), that methylamine had no effect on transferrin endocytosis (Figure 3) or steady-state binding (Table I). In the presence of increasing concentrations of methylamine, the release of transferrin was progressively slowed (Table I; Figure 4) and an increasing proportion of the released transferrin was in the diferric form (Figure 5B–D). At all concentrations of methylamine (most markedly at 25 mM) the first reincubation contained up to 60% of apotransferrin and the monoferric species. The finding that these iron-depleted species rapidly diminished with time suggests that they were preferentially lost from the cells. An explanation of how this occurs could be that in the methylamine-treated cells the occupied receptor continues to cycle to the cell surface and the monoferric transferrin and apo-

transferrin, which have a lower affinity for the transferrin receptor than does diferric transferrin (Young et al., 1984), are lost from the cell surface at a greater rate than diferric transferrin.

Despite the evidence that removal of iron from transferrin both in vitro (Ankel & Petering, 1981) and in vivo (Thorstensen & Romslo, 1984) may involve a reductive process, the evidence has been mainly interpreted as indicating that iron is removed from transferrin by cells by protonation of the iron-binding sites in an intracellular acidic environment (Karin & Mintz, 1981; Morgan, 1981; van Renswoude et al., 1982; Klausner et al., 1983a). In vitro the two iron-binding sites of transferrin have different susceptibilities to proton attack (Princiotto & Zapolski, 1975; Lestas, 1976). At pH 7.4 the N-terminal site has an affinity for iron of about $^1/_5$th that of the C-terminal site, while at pH 6.7 the disparity increases, with the affinity of the N-terminal site being only $^1/_{20}$th that of the C-terminal site. Below pH 5 both sites rapidly lose the ability to bind iron. This pH is close to that found within endocytic vesicles (Okhuma & Poole, 1978; Poole & Okhuma, 1981), a condition that should lead to the release of both iron atoms from transferrin. Direct estimation by fluorescence quenching of the pH encountered by transferrin within cultured cells has indicated a range of pHs for the endocytic vesicle. While van Renswoude et al. (1982) found that transferrin passes through a pH of 5.5 in the K562 cell, Yamashiro et al. (1984) suggest that the pH does not drop below 6.5 in the Chinese hamster ovary cell. Because the relative affinity of the iron-binding sites of transferrin is so markedly different at the latter pH, the release of large amounts of the acid-stable monoferric transferrin might be expected. While we found that, with control cells, consistently more C-terminal monoferric transferrin was released (C/N = 1.3), suggesting that there is a slight preference for the iron in the acid-labile N-terminal binding site, this difference was not sufficient to suggest that a pH as high as 6.5 was encountered in the K562 cell. The finding that the major species of transferrin in human plasma, in which the transferrin was 30% saturated with iron, were apotransferrin and the C-terminal monoferric transferrin also suggests that iron carried by the N-terminal monoferric transferrin may be preferentially available to cells under physiological conditions (Evans & Williams, 1978). This conclusion was reached by van Baarlen et al. (1980) after they had found that the predominant monoferric transferrin appearing in incubations of rabbit reticulocytes with diferric transferrin was the C-terminal species.

In our experiments, under conditions in which the pH of the endocytic vesicles was disturbed by exposing the cells to increasing concentrations of methylamine, we hoped to amplify the differences in the affinity of iron for the two sites within the cell. We found that at all concentrations of methylamine, maximally at 10 mM, the C-terminal or "acid-stable" monoferric transferrin was preferentially generated by the cells (Figure 6). It is unlikely that both of the monoferric species were generated in equal amounts, with the C-terminal being released preferentially, since we have recently shown that the two monoferric transferrins have the same affinity for the transferrin receptor on rabbit reticulocytes (Young et al., 1984) and so would be expected to be released at the same rate. Although the monoferric species were not the major form of the transferrin generated by the cells (Figure 5), since apotransferrin predominated under control conditions and diferric transferrin predominated when methylamine was present, it is likely that the ratio of the two monoferric species reflected the pH to which the transferrin had been exposed in the endocytic vesicle. We have extrapolated from the data of Princiotto & Zapolski (1975), who found that in vitro different proportions of the monoferric transferrins were generated on the addition of iron to human apotransferrin, depending on the pH of the incubation medium. These authors found that at pH 5.0 the ratio of the two species was 1.25, a figure similar to that found with the control cells in the present study. The pH required to generate a ratio (C/N) of 2 (that found in the presence of 5 mM methylamine) was 5.4, while that required to generate a ratio of 2.4 (seen with 10 mM methylamine) was about 5.9. Above pH 6 they found that the ratio fell as the pH effect on iron binding at the two sites became less marked, and in agreement with this, we found that the C/N ratio fell to less than 2 in the presence of 25 mM methylamine. Poole & Ohkuma (1981) have shown that weak bases produce a concentration-dependent rise in the pH of lysosomes, and if, as seems likely, this effect also occurs in endocytic vesicles, we suggest that the release of varying proportions of the C- and N-terminal monoferric transferrins is caused by the increasing concentration of methylamine, inducing a progressive evaluation in the pH of the endocytic vesicles. This is direct evidence that protonation is of primary importance in the removal of iron from the two iron-binding sites of transferrin in cells.

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^{13}C NMR Study of the Ionizations within a Trypsin–Chloromethyl Ketone Inhibitor Complex†

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ABSTRACT: ^{13}C NMR is used to detect ionizations within a trypsin–chloromethyl ketone inhibitor complex. The pK_a values observed are compared with those predicted by free-energy relationships. For the denatured/autolyzed inhibitor complex, a $pK_a = 5.26$ is observed, which is assigned to the ionization of the imidazole of histidine-57. For the intact inhibitor complex a $pK_a = 7.88$ is determined. This pK_a is assigned to the ionization of the hemiketal hydroxyl ($pK_a = 7.88$–8.1) and provides the first direct evidence that the serine proteases are able to stabilize the oxyanion of tetrahedral adducts. Indirect evidence is adduced that the imidazole pK_1 of histidine-57 is ≥ 8.1. Line-broadening studies suggest that there may be extra fast exchange line broadening, which could result from rapid tautomeric exchange between neutral and zwitterionic species within the inhibitor complex. The significance of these results for the catalytic mechanism of serine proteases is discussed.

It is generally assumed that proteolysis reactions catalyzed by the serine proteases proceed via tetrahedral addition compounds formed prior and subsequent to the acylation and deacylation reactions. Although a tetrahedral intermediate (THI)[1] should not accumulate during catalysis (Mackenzie et al., 1984), its stabilization could account for much of the catalytic efficiency of proteases. Indeed, recent kinetic studies suggest that transition-state stabilization of the oxyanion of a THI is essential for the serine proteases (Asboth & Polgar, 1983). Considerable effort has therefore been exerted in the development of transition-state analogues that will mimic such intermediates in the hope that such studies will provide evidence of how enzymic stabilization of the THI is achieved.

Hydrogen bonding of the oxyanion of the THI was one of the first mechanisms to be proposed (Henderson, 1970). Stabilization of the oxyanion by interaction with cationic histidine-57 was first discussed by Caplow (Caplow, 1969; Lucas et al., 1973), who suggested that such an interaction would lower the pK_a of histidine-57. Considerable evidence

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[1] Abbreviations: Z-Lys-CMK, 1-chloro-3-(carbobenzyloxyamino)-7-aminoheptan-2-one; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; LEFS, uniform-field linear electric field shift; LB, line broadening; FID, free-induction decay; NMR, nuclear magnetic resonance; T, tesla; THI, tetrahedral intermediate.

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