Binding of transferrin-iron by adriamycin at acidic pH

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It is shown that adriamycin is able to chelate iron released from iron-loaded serum transferrin in the pH range from 6.5–4.1. The kinetics of iron transfer to free adriamycin and to adriamycin covalently attached to the transferrin has been determined. The results show that adriamycin, if introduced into intracellular acidic compartments, could function as acceptor for transferrin-iron.

Adriamycin Transferrin (Human serum) Adriamycin-Fe^{3+} complex

1. INTRODUCTION

Adriamycin (doxorubicin), an anthracycline antibiotic with antitumor activity, interacts with Fe^{3+} forming complexes of considerable stability [1,2]. There is evidence from several in vitro studies [3–11] that these drug-metal ion complexes can act as redox catalysts in the formation of reactive and toxic oxygen species. In a previous report [12] we showed that ADM in vitro is capable of binding iron from the iron storage protein ferritin. We have extended this work to the interaction between ADM and the serum glycoprotein Tr involved in iron transport.

2. MATERIALS AND METHODS

2.1. *Preparation of iron-loaded transferrin*

Iron-loading of human serum transferrin was carried out with ferric nitrilotriacetate in the presence of carbonate [13]. Nitrilotriacetate and excess iron was removed by passage of Fe-Tr through a 25 × 2.5 cm Sephadex G-25 column equilibrated and eluted with 0.1 M $NaClO_4$ [13, 14], followed by dialysis at 4°C for 3 days against 3 changes of 500 ml of 20 mM Hepes (pH 7.4). Fe-Tr concentration was determined at 280 nm using $A_{1\,cm}^{1\%} = 14.1$ [15] and an M_r for Tr of 79600 [16]. Total iron in Fe-Tr was determined after sulfuric acid digestion as described in [17]. The total iron content was 1.87 ± 0.04 mol Fe/mol Tr (mean ± SE of 3 preparations). From the absorbance at 465 nm [18] an iron content of 1.95 ± 0.01 mol Fe/mol Tr was calculated.

Abbreviations: ADM, adriamycin; Tr, transferrin; Fe-Tr, iron-loaded transferrin

2.2. *Determination of iron transfer to ADM*

Formation of ADM-Fe^{3+} complex with Fe-Tr as iron donor was monitored spectrophotometrically using the appearance of an absorption band at 600 nm for ADM upon binding to Fe^{3+} [5]. The reactions were performed in 1-cm light-path cuvettes thermostatted to 37 ± 1°C and the reaction mixtures contained 500–550 μl of 150 mM NaCl, 50 mM Tris-acetate buffer titrated with HCl to various pH values, 0–30 μl of 5 mM ADM/HCl in water and 25–50 μl Fe-Tr or 100–150 μl ADM-(Fe-Tr) conjugate added as the last component.

2.3. *Materials*

Human serum transferrin (98%, substantially iron-free) and Na_3nitrilotriacetate were from Sigma (St. Louis, MO). Na_2ADP and Na_2ATP were obtained from Boehringer (Mannheim) and

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dithiobis(succinimidyl propionate) from Pierce (Illinois, USA). Adriamycin/HCl was a gift from Farmitalia Carlo Erba (Milano, Italy).

3. RESULTS AND DISCUSSION

Time courses for formation of 600 nm light-absorbing ADM-Fe^{3+} complex during incubation on ADM with Fe-Tr in KCl-Tris-acetate buffers at various pH values are given in fig.1A. Complex formation occurred at increasing rates when pH was lowered from 6.5 to 4.6. In the same pH range, iron dissociated from the iron binding sites of the Tr as judged by the disappearance of 465 nm absorbance during incubation of Fe-Tr in the absence of ADM (fig.1B). Formation of ADM-Fe^{3+} complex appeared to be transient at pH values below 4.6. Using $\epsilon = 11400\ M^{-1}\cdot cm^{-1}$ for the $(ADM)_3$-Fe^{3+} complex at 600 nm [5] and $\epsilon = 4800\ M^{-1}\cdot cm^{-1}$ for diferric Tr at 465 nm [18] the initial rates for formation of ADM-Fe^{3+} complex at the different pH values were estimated to be about equal to or lower than the initial rates of loss of iron from Fe-Tr. Thus, the observed binding of Tr-iron by ADM can be explained by chelation of the iron which dissociates from the Tr at acidic pH. The finding that the initial rates for development of 600 nm absorbance were almost independent of ADM concentration when present in molar excess to Tr-iron is consistent with this mechanism (fig.2). The plateau values for 600 nm absorbance reached at the different ADM concentrations are given in the inset of fig.2. About 45% of the total iron in Fe-Tr was estimated to be complexed by ADM at 100 μM.

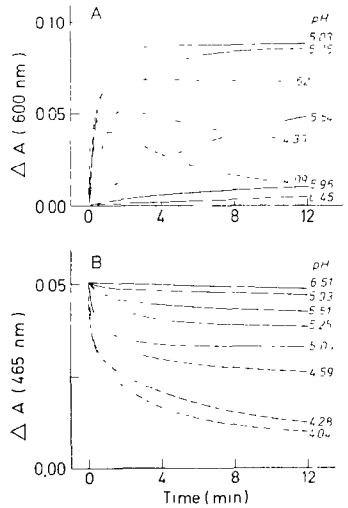

Fig.1. (A) Time courses for formation of 600 nm absorbance during incubation of iron-loaded transferrin with adriamycin. Transferrin (10.6 μM), adriamycin (69.4) μM) and buffer-pH values as indicated. (B) Loss of iron from iron-loaded transferrin monitored at 465 nm during incubations in the absence of adriamycin. Transferrin (10.8 μM) and buffer-pH values as indicated.

The binding by ADM or iron released from Fe-Tr at acidic pH was inhibited by the iron chelators EDTA, nitrilotriacetate, phosphate and ATP whereas ADP appeared to facilitate iron transfer to ADM (table 1). ADP was also able to counteract the inhibitory effect of phosphate. The reason for this may be found in the ability of ADP to form stable ternary complexes with ADM and Fe^{3+} [3]. Ca^{2+} and Mg^{2+} had no significant effect on the kinetics of iron binding by ADM.

The kinetics of iron transfer to ADM covalently attached to Fe-Tr was also determined. ADM was conjugated to Fe-Tr by linking the sugar amino group of ADM to amino groups in the protein with the bifunctional reagent dithiobis(succinimidyl propionate) [19] leaving the metal ion binding capacity of the anthracycline moiety of ADM intact (in preparation). The pH threshold for iron transfer to the protein-bound ADM was found similar to that for transfer to 'free' ADM (pH 5.5–5.6), but the rates of iron transfer ob-

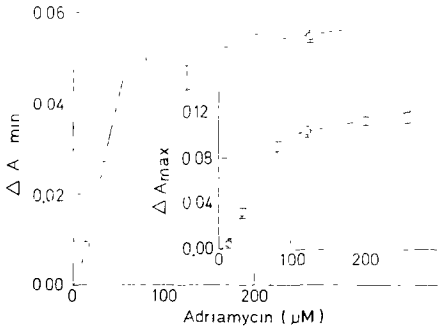

Fig.2. Initial rates and plateau values (inset) for development of 600 nm absorbance during incubation of iron-loaded transferrin (10.6 μM) with adriamycin at different concentrations (14.1–253 μM). Buffer pH (5.23–5.25). Data are presented as the mean ± SE from 3 separate experiments.

Table 1

Effect of various factors on the kinetics for binding of transferrin-iron by adriamycin

Incubation system	Initial ΔA/min	ΔA_{max}
ADM + Fe-Tr	0.027 ± 0.003	0.108 ± 0.017
+ EDTA (0.2 mM)	–	0.001 ± 0.001
+ nitrilotriacetate (0.2 mM)	–	0.001 ± 0.001
+ phosphate (1 mM)	0.020 ± 0.011	0.011 ± 0.002
+ ATP (1 mM)	0.018 ± 0.004	0.020 ± 0.002
+ ADP (1 mM)	0.082 ± 0.003	0.097 ± 0.002
+ ADP (1 mM) + phosphate (1 mM)	0.092 ± 0.018	0.093 ± 0.010
+ Ca^{2+} (1 mM)	0.030 ± 0.002	0.107 ± 0.002
+ Mg^{2+} (1 mM)	0.034 ± 0.009	0.117 ± 0.007

Formation of adriamycin-Fe^{3+} complex was followed at 600 nm during incubation at 37°C. Iron-loaded transferrin (17.5 μM), adriamycin (80.1 μM) and pH (5.30–5.38). Results are mean ± SE, $n = 3$–5

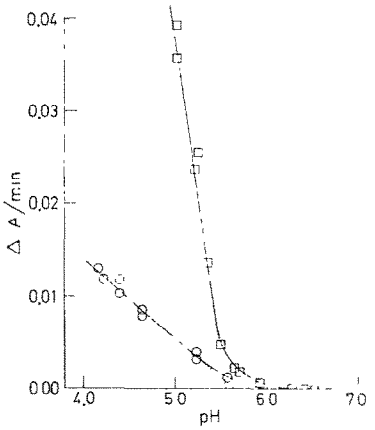

Fig.3. Effect of pH on the initial rates of Fe^{3+} transfer from transferrin to free adriamycin (□) and to adriamycin conjugated to transferrin (○). (□) Transferrin (5.30–5.34 μM) and adriamycin (69.7 μM). (○) Transferrin (5.11–5.20 μM) and transferrin-bound adriamycin (70.6–71.7 μM).

tained in the conjugates were 4–5-times slower than measured with 'free' ADM (fig.3). This may be due to the low diffusion coefficient of the protein-fixed ADM compared to that of 'free' ADM or to steric hindrance to iron binding by ADM on the surface of the protein.

There is evidence both from microscopic and biochemical studies, that cells can obtain iron by receptor-mediated endocytosis of Fe-Tr [20]. The low affinity of Tr for iron at acidic pH [21,22] suggests that iron is released from the internalized receptor-(Fe-Tr) complex during its passage through acidic cell compartments [23,24]. This study shows that ADM has the capacity for chelating iron which dissociates from Fe-Tr at acidic pH. The results therefore suggest that ADM, if introduced into Fe-Tr containing acidic compartments, could act as a 'false' acceptor for iron. ADM is a membrane permeable weak base and can be expected to concentrate in acidic cell structures by a simple diffusion process [25,26]. A recent study [27] indicates that iron is accessible to chelation by 2,2′-bipyridine and phenanthroline immediately after release from Fe-Tr in intracellular vesicles. It is clear, also from the data in table 1, that the efficiency of ADM as an acceptor for Tr-iron in cell organelles will depend on the properties and affinity of other potential iron chelating agents present. The finding that ADM can exchange iron with Fe-Tr at acidic pH also after covalent attachment to Tr opens the possibility for specific studies on intracellular iron chelation by ADM. Membrane binding and endocytosis of ADM-(Fe-Tr) conjugates are presently being investigated.

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