Biochemical Pharmacology, Vol. 57, pp. 1031–1035, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00014-3

SHORT COMMUNICATION

Competition between Iron(III)-selective Chelators and Zinc-finger Domains for Zinc(II)

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ABSTRACT. Many iron(III)-selective chelators possess an appreciable affinity for zinc(II) and this can prove to be undesirable when such chelators are being assessed for clinical application. At present, there is no useful test available which can reliably access this problem. In the present manuscript, we provide evidence that indicates that a zinc-finger protein MTF-1, (metal transcription factor-1) may prove to be a suitable candidate. *N,N′*,2-hydroxybenzyl ethylenediamine diacetic acid, in contrast to desferrioxamine, removes zinc quite efficiently from MTF-1. BIOCHEM PHARMACOL **57**;9:1031–1035, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. iron chelators; zinc; zinc-fingers; MTF-1

There is considerable pressure to design novel iron(III)-selective chelators, which are orally active and have potential to treat iron overload, as found in patients who are dependent on regular blood transfusions, for instance thalassaemia major patients [1, 2]. DFO§ is used clinically at the present time, but unfortunately lacks oral activity [2]. Hexadentate iron(III) chelators are ideal for scavenging iron under biological conditions, as they tend to form kinetically inert complexes and therefore do not redistribute iron [3]. Unfortunately, it is difficult to design orally active hexadentate ligands, as they tend to possess molecular weights in excess of 500 and above this size, non-facilitated absorption by the gastrointestinal tract is low [4]. There is an exception to this general statement, namely the aminocarboxylate family, which includes EDTA and HBED (Fig. 1). Some members of this group possess molecular weights <400 (Table 1). The smallest hexadentate ligand of this class is EDTA, which forms a basket-type complex, thereby creating a seventh ligand site [5]. This site is capable of binding small ligands such as hydrogen peroxide, and hence the iron complex is toxic [6]. The closely related HBED, by virtue of the two phenolic functions, possesses a higher affinity constant for iron(III), namely 10^{-40}M [7, 8], and this reduces the toxicity of HBED. The reason for the relatively small molecular size of EDTA and HBED is that two nitrogen atoms form part of the structural framework of the ligand, as well as acting as ligating atoms. Charged oxygen atoms, which are favoured iron(III) ligands [9], lack this ability. The presence of the two co-ordinating nitrogen atoms in EDTA and HBED also leads to an appreciable affinity for zinc (Table 1) [8]. Although the selectivities of DFO and HBED for iron(III) vs zinc(II) as indicated by the ΔpM values are similar (Table 1), HBED binds zinc(II) over one thousand times more tightly than DFO. The zinc cation is a critical component of many proteins, serving both catalytic and structural roles, and consequently it is undesirable for iron chelators to possess an appreciable affinity for zinc. Since zinc-fingers are probably the most widely distributed class of transcription factors, many hundreds having been characterised [11], we decided to investigate whether HBED and other iron(III) chelators could compete with zinc-finger proteins for zinc.

One zinc-finger protein we examined in these studies binds specifically to metal regulatory elements in the MT promoter and transactivates MT gene expression in response to metals [12] and oxidative stress [13]. This protein, termed MTF-1, is a six Zn-finger transcription factor in the Cys_2His_2 family [12]. MTF-1 plays an essential role in basal, as well as heavy metal-induced MT gene expression [14]. Zinc metabolism is at least partially regulated by metallothioneins, as they are indeed the most abundant intracellular, high-affinity Zn-binding proteins in higher eukaryotes. They have been shown to be important in zinc homeostasis through the study of knockout mice and mice which overexpress MT [15, 16]. Overexpression of MT protects, whereas a lack of MT exacerbates the physiological effects of dietary zinc deficiency. MT genes contain metal response elements in the proximal promoter which play a central role in transcriptional activation of these genes in response to metals. The transcription factor MTF-1 contains six Zn-fingers of the Cys2His2 type and is

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§ *Abbreviations:* DFO, desferrioxamine; HBED, *N,N′*,2-hydroxybenzyl ethylenediamine diacetic acid; MTF-1, metal transcription factor-1; Sp1, a three Zn-finger transcription factor; MT, metallothionein; and TNT, Reticulocyte lysate coupled transcription and translation test.

Received 19 June 1998; accepted 12 November 1998.

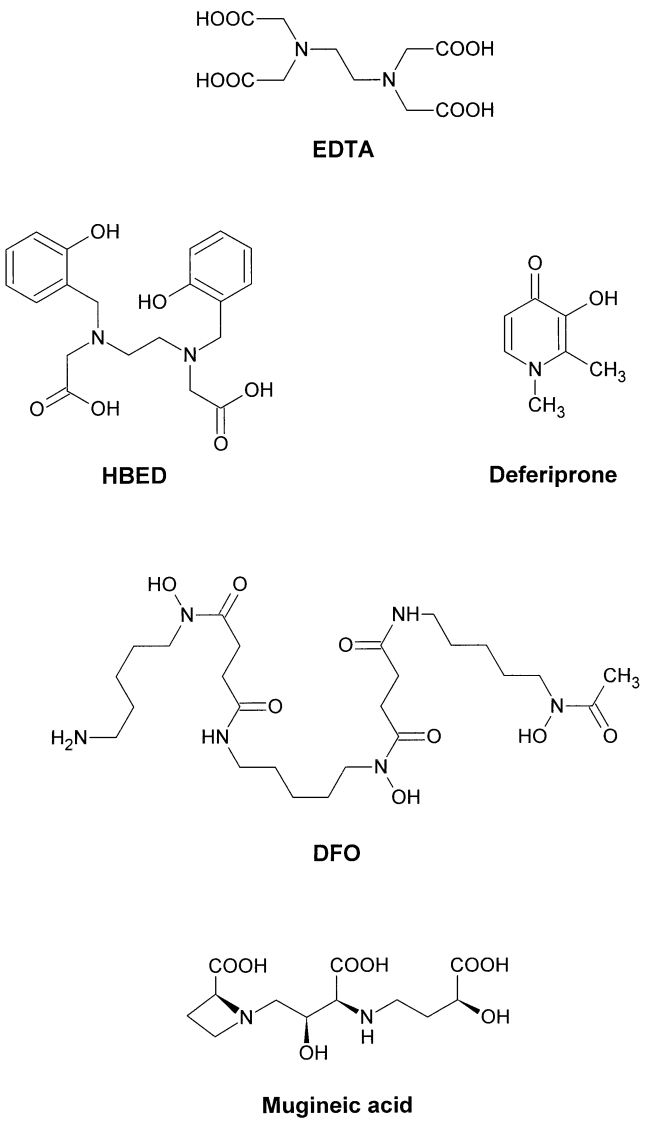

FIG. 1. Structures of iron(III)-selective ligands.

required for basal, as well as zinc-induced MT expression [14]. MTF-1 is apparently unique among Zn-binder transcription factors in that it is induced to bind to DNA by zinc concentrations that are in the μM range [17, 18]. Therefore, MTF-1 can be considered to be a metalloregulatory protein which governs MT gene expression in response to zinc. Furthermore, embryos in which both alleles of MTF-1 have been disrupted die at day 14 of development due to failure in liver development [19], which underscores the importance of MTF-1. Thus, we selected MTF-1 as a suitable protein to test the ability of a range of potential iron(III) chelators to interfere with the biological properties of zinc-finger proteins. In addition, we examined the effects of chelators on the DNA-binding activity of the constitutively expressed protein Sp1 [20, 21]. Sp1 is a three Zn-finger transcription factor in the Cys_2His_2 family, the DNA-binding activity of which is not influenced by exogenous zinc and less susceptible to inactivation by EDTA or dilution *in vitro* [22].

TABLE 1. Affinities of iron(III) ligands for both iron(III) and zinc(II) [10]

Chelator	pFe^{III}	pZn^{II}	ΔpM (pFe^{III}–pZn^{II})	Molecular weight
Deferiprone	19.5	6.5	13.0	139
HBED	31.0	9.5	21.5	388
EDTA	23.0	14.5	8.5	292
DFO	26.5	6.0	20.5	560
Mugineic acid	16.8	10.4	6.4	320

pM is defined as $-\log[M]$ where [M] is the concentration of the hydrated cation at pH 7.4, when $[M]_{TOTAL} = 10^{-6}$M and $[L]_{Total} = 10^{-5}$M.

MATERIALS AND METHODS

Materials

DFO and HBED were supplied by Novartis. Deferiprone was synthesized as previously reported [23]. EDTA was purchased from Sigma Chemical Company. The TNT kit was purchased from Promega Corporation.

In Vitro *Transcription/Translation of Mouse Recombinant MTF-1 and Sp1*

The cDNAs for mouse MTF-1 and Sp1 were as described in detail previously [16, 22]. Recombinant MTF-1 and Sp1 were synthesized *in vitro* using a TNT system (Promega Biotech), containing 1 μg of the MTF-1 or Sp1 plasmids and Sp6 or T7 RNA polymerase, respectively, according to the manufacturers' directions.

Electrophoretic Mobility Shift Assay

The assay was performed as described previously [18, 22]. The DNA-binding of MTF-1 was activated by adjusting the exogenous zinc concentration of the TNT lysate to 60 μM and incubating at 37° for 15 min. Sp1 was treated in this fashion, although this was not required to activate Sp1 DNA-binding activity. One μL of activated lysate (of a 50 μL TNT reaction) was transferred to buffer containing 12 mM HEPES (pH 7.9), 60 mM KCl, 0.5 mM dithiothreitol, 12% glycerol, 5 mM $MgCl_2$, 0.2 μg dI/dC/μg protein, 2–4 fmol end-labelled double-stranded oligonucleotide (5000 cpm/fmol) in a total volume of 20 μL on ice. Effects of the addition of chelators (EDTA, HBED, DFO, and deferiprone) on MTF-1- or Sp1-binding activity were examined as indicated in the figure legends. The oligonucleotide sequence binding sites used were as described [18, 22]. The metal regulatory element oligonucleotide (GATCCAGG GAGCTCTGCACACGGCCCGAAAAGTA) represents a high-affinity binding site for MTF-1, whereas the Sp1 oligonucleotide (GGCCGGGGCGGGTAGGGGCCGG GGCGGGTAGGTCTAG) represents a high-affinity binding site for Sp1. Protein–DNA complexes were separated at 4° using 4% polyacrylamide gel (acrylamide: bisacrylamide/80:1) electrophoresis. The gel was dried and labelled complexes were detected by autoradiography.

RESULTS

The DNA-binding activity of MTF-1 is reversibly activated by μM concentrations of zinc [22]. We previously demonstrated that Zn-activated MTF-1 lost the ability to bind DNA when it was diluted into an electrophoretic mobility shift assay reaction, such that the zinc concentration was below 0.5 μM and the reaction was warmed to 37° for 1 hr. The DNA-binding activity could be completely restored by raising the zinc concentration above 15 μM. In this study, MTF-1 produced *in vitro* was activated as described in Materials and Methods, followed by 20-fold dilution into the reaction buffer. The final zinc concentration was 2 μM. Under these conditions, approximately 50% of total DNA-binding capacity was retained after incubation at 37°. When the DNA-binding capacity was examined in the presence of the aminocarboxylate compounds, the band intensity due to the formation of the protein/DNA complex was significantly reduced as compared to an identical reaction without chelator (Fig. 2A). This reduction in band intensity was much less dramatic with Sp1, which is markedly less dependent on exogenous zinc for DNA-binding activity than MTF-1 [13] (Fig. 2B). The bulk of

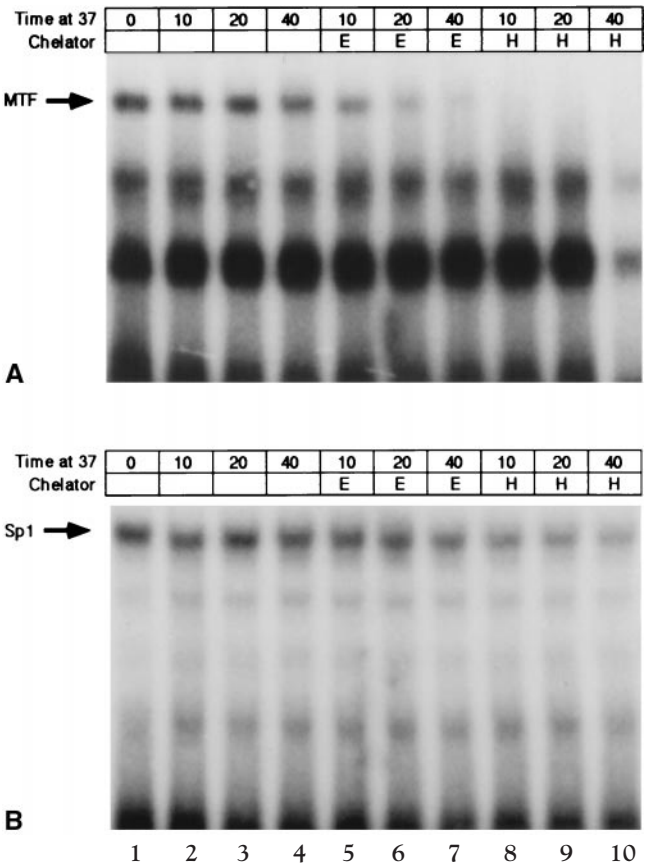

FIG. 2. Electrophoretic mobility shift assay using activated TNT lysate incubated with chelator. One μL of an activated TNT lysate from a reaction programmed with MTF-1 or Sp1, (2a and 2b respectively) were incubated without chelators (lanes 2–4) or with 50 μM of EDTA (E) (lanes 5–7) or 50 μM HBED (H) (lanes 7–10) for the indicated times and electrophoresed followed by autoradiography.

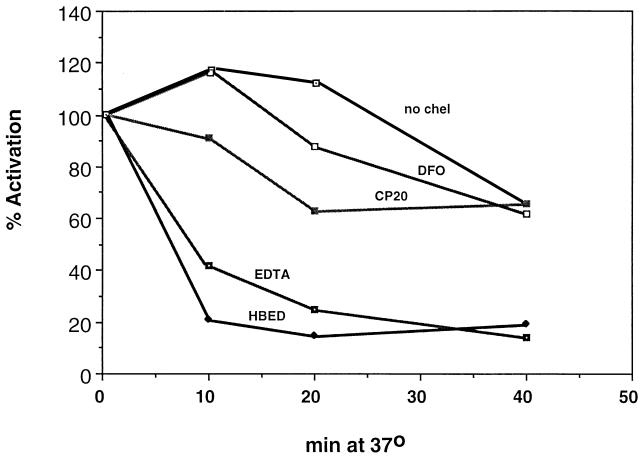

FIG. 3. Comparison of chelator inactivation of MTF-1 DNA-binding. Electrophoretic mobility shift assay using activated TNT lysate incubated without chelator or with DFO, deferiprone (CP20), EDTA, or HBED. One μL of an activated TNT lysate from a reaction programmed with MTF-1 was incubated for the indicated time at 37° and returned to ice. Labelled oligo was added and the binding reaction proceeded for 15 min followed by PAGE. The gels were dried and band intensity was determined using phosphoimage analysis (Molecular Dynamics). The activation percentage is compared to activated MTF-1, which was kept on ice after activation.

this inhibitory effect occurred within 10 min when MTF-1 was incubated with chelators at 37° (Fig. 3). Although there was a slight inhibitory effect induced by the iron-specific chelators desferrioxamine and deferiprone (Fig. 3), this effect was much less marked than that induced by the two aminocarboxylate ligands. Dose–response curves for these latter two ligands, after incubation with MTF-1 for 20 min, demonstrate that HBED was found to be a more potent inhibitor of zinc-induced MTF-1 binding activity than EDTA (Fig. 4). Furthermore, even at clinically attainable levels (5 and 10 μM), HBED was found to exert a potent inhibitory influence on MTF-1-binding activity.

DISCUSSION

Clearly, when compared with desferrioxamine and deferiprone (a hydroxypyridinone), HBED presents a disadvantage, as at clinically attainable levels it is able to compete effectively with MTF-1 for zinc. As MTF-1 is likely to be intimately involved with the control of intracellular zinc levels, this is an undesirable feature for a supposedly iron-selective chelator.

Although the competition studies undertaken in this study were made under non-physiological conditions, it is known that some iron chelators remove zinc during clinical use, and it was for this reason that the closely related aminocarboxylate ligand, diethylene triamine penta-acetic acid (DTPA), was removed from clinical trials [24]. Thus, even under iron overload conditions, when non specifically bound iron is available, zinc will be scavenged by so called iron-selective ligands, should the ligands possess a suffi-

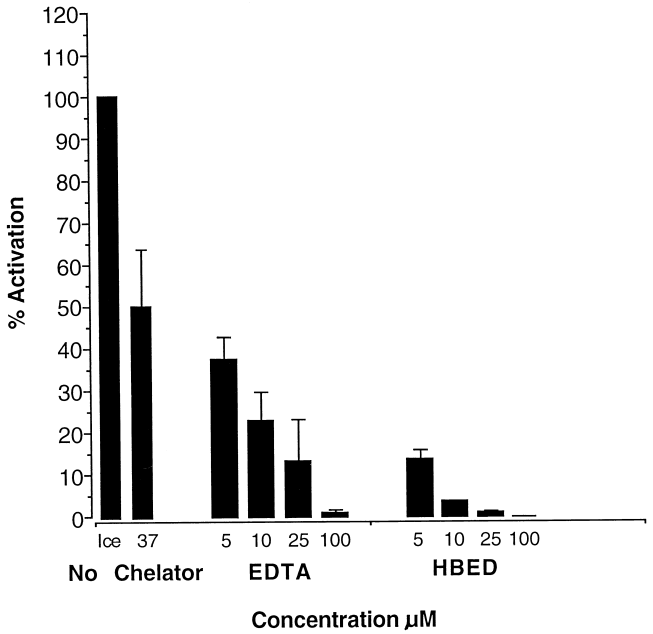

FIG. 4. Titration of chelator and its effect on inactivation of MTF-1 DNA-binding. Electrophoretic mobility shift assay using activated TNT lysate incubated with chelator at the indicated concentration for 20 min at 37°. Labeled oligo was added and the binding reaction proceeded for 15 min followed by PAGE. The gels were dried and band intensity was determined using phosphoimage analysis (Molecular Dynamics). The activation percentage is compared to activated MTF-1 with no incubation at 37°. The results are presented as standard errors of the mean (N = 3).

ciently high affinity for zinc(II). The lower affinity for zinc(II), demonstrated by desferrioxamine and deferiprone, is due to the chemical nature of these ligands; they both utilize oxygen atoms with a high charge density as chelating functions. In contrast, with both EDTA and HBED, two of the six coordinating atoms are nitrogen and this favours interaction with divalent cations, including zinc(II). Significantly, mugineic acid (Fig. 1), a naturally occurring aminocarboxylate, is involved in the transport of both iron(III) and zinc(II) in plant roots [25, 26].

Interference with zinc metabolism is an undesirable property for clinically useful iron chelators [1, 3], and therefore the MTF-1-binding assay, as described in this communication, provides a useful initial toxicology screening method for such compounds. We are currently investigating the influence of a wide range of hydroxy pyridinones in this system.

This study was supported, in part, by a grant from the NIH to G. K. A. (ES05704). An NRSA fellowship provided support for D. B. (F32 ES05753-01).

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