THE BASIS FOR EDTA-STIMULATION OF METHEMOGLOBIN REDUCTION
IN HEMOLYSATES OF HUMAN ERYTHROCYTES

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Summary: We have studied the stimulation by EDTA of methemoglobin reduction
in hemolysates of human erythrocytes. The EDTA effect has been shown not to be
the result of an allosteric interaction of EDTA with hemoglobin or the result
of a photochemical reduction. The effect does not appear to be due to a direct
interaction of free EDTA with either of the catalytic components of the
erythrocyte methemoglobin reduction system. The EDTA stimulation seen in hemo-
lysates is due to the formation of an iron-EDTA complex, which transfers
electrons from the reductase to methemoglobin.

In a previous study (1) we observed that 0.5 m$\underline{M}$ EDTA stimulates methemoglo-

bin reduction 60 to 70% in hemolysates of human erythrocytes. Stimulation of

methemoglobin reduction by EDTA has been reported previously (2,3). There are

several plausible explanations for the observed stimulations. The effect may

be due to a direct binding of EDTA to methemoglobin. EDTA acts as an allosteric

effector of hemoglobin (4), as do organic phosphates such as DPG*** and IHP (5).

Binding of polyanions also causes conformational changes in methemoglobin (6-8)

and alters its oxidation-reduction equilibria (9,10). Moreover, IHP increases

the rate of methemoglobin reduction by purified erythrocyte NADH-dependent

reductase in the absence (11,12) or the presence (13) of cytochrome $\underline{b}_5$. DPG

stimulates methemoglobin reduction by ascorbic acid (14). A second possible

explanation for the stimulation would be an EDTA-dependent photochemical re-

duction of one of the components of the methemoglobin reduction system. EDTA

and flavin have been shown to be an effective system for the photoreduction of

many oxidation-reduction proteins (15). Thirdly, the stimulation might be a

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*** Abbreviations used are DPG, 2,3-diphosphoglycerate; and IHP, inositol
     hexaphosphate.

direct action of EDTA on soluble NADH-dependent cytochrome b_5 reductase or

soluble cytochrome b_5, the catalytic components of the erythrocyte methemoglobin

reduction system. EDTA is known to stabilize the erythrocyte reductase, and

therefore has been added in buffers used for its purification (16,17).

Lastly, EDTA may be acting as a chelating agent either to remove an

inhibiting cation or to form a complex which is capable of undergoing oxida-

tion reactions. A candidate for a redox active complex would be an iron-EDTA

complex; this complex has already been reported to serve as a substrate for

microsomal NADH-cytochrome b_5 reductase (18). The ferrous-EDTA complex re-

sulting from such a catalysis might then reduce cytochrome b_5 or methemoglobin.

This paper presents evidence that the stimulation of methemoglobin reduction

in hemolysates observed upon the addition of EDTA is the result of chelation

of free iron present in the hemolysate with subsequent coupling of electron

transfer between reductase and methemoglobin by this iron-EDTA complex.

MATERIALS AND METHODS

Outdated human blood was donated by the University of Michigan Medical Center Blood Bank. Blood was stored in citrate-phosphate-dextrose (3.20 g citric acid, 25.8 g sodium citrate, 25 g glucose, and 2.18 g $NaH_2PO_4 \cdot H_2O$ in 1 liter of water), and was used within 1 week of the expiration date. Methemoglobin and methemoglobin-containing erythrocytes were prepared as previously described (1). Bovine erythrocyte cytochrome b_5 was purified by the method of Douglas and Hultquist (19). Human erythrocyte cytochrome b_5 reductase was purified by a modification of the method of Kuma and Inomato (20). NADH was purchased from Sigma Chemical Co. EDTA and $FeCl_2$ were purchased from Matheson, Coleman, and Bell.
Methemoglobin reduction in hemolysates was assayed as previously des-cribed (1). The reaction in hemolysates proceeds linearly almost until comple-tion. Methemoglobin reduction in a reconstituted system was carried out with final concentrations of 100 μM NADH, 0.01 μM human erythrocyte cytochrome b_5 reductase, 142 μM human methemoglobin, 0.005 mM EDTA, 0.025 M bis-Tris, pH 7.2, and, when added, 0.1 μM bovine erythrocyte cytochrome b_5 or 4 μM $FeCl_2$. When indicated, EDTA concentration was increased to 0.5 mM. The reactions were monitored at 37°C by following the increase in absorbance at 577 nm. The linear initial velocities were measured. All rates and concentrations of methemoglobin are expressed on the basis of heme as described previously (1).

RESULTS AND DISCUSSION

Fig. 1 shows that at low concentrations, DPG has no effect on the rate of

methemoglobin reduction in hemolysates, and IHP has only a small (but repro-

ducible) stimulation. At the same concentrations, EDTA shows a relatively large

(1.6- to 1.7-fold) stimulation of initial velocity. If EDTA stimulation were
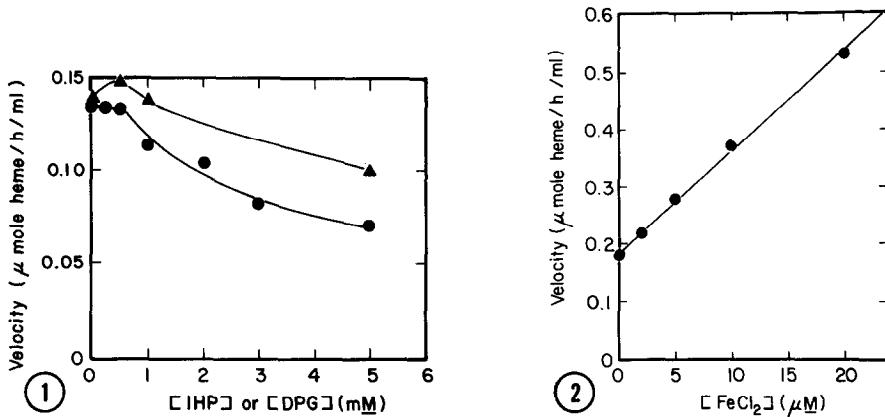
Fig. 1. Effect of IHP (▲) and DPG (●) on the reduction of methemoglobin in hemolysates made by a 1:4 dilution of packed red blood cells with water. For details, see Materials and Methods.

Fig. 2. Effect of $FeCl_2$ concentration on the rate of methemoglobin reduction in a hemolysate made by a 1:4 dilution of packed red blood cells with water. The hemolysate contained 0.5 m$\underline{M}$ EDTA. For details, see Materials and Methods.

the result of allosteric regulation, such as occurs with IHP and DPG, then IHP and DPG would have been expected to show stimulations similar to that seen with EDTA. The lack of such stimulation by IHP and DPG suggests that the EDTA stimulation is probably not solely due to EDTA acting as an allosteric regulator of hemoglobin. Higher concentrations of IHP and DPG caused an inhibition of methemoglobin reduction, presumably by an ionic strength effect (1). Even at low concentrations, these polyanions make a large contribution to ionic strength due to their multiple charges.

Stimulation by EDTA of methemoglobin reduction in hemolysates was the same in the dark as in the light, demonstrating that the stimulation was not due to a photochemical reduction reaction.

In the presence of EDTA, $FeCl_2$ further stimulated methemoglobin reduction in the hemolysate. The rate of methemoglobin reduction in a hemolysate containing 0.5 m$\underline{M}$ EDTA was found to be linearly related to the concentration of added $FeCl_2$ from 0 to 20 $\mu\underline{M}$ $FeCl_2$ (Fig. 2), whereas addition of $FeCl_2$ to a hemolysate containing no EDTA had no effect on the rate of reduction up to a concentration of 200 $\mu\underline{M}$ $FeCl_2$.

TABLE 1

Effect of $FeCl_2$ and Cytochrome $\underline{b}_5$ on the Rate of Methemoglobin Reduction

in a Reconstituted System Containing EDTA

Additions	Rate (μmole heme/hr/ml)
Cytochrome $\underline{b}_5$	0.034
$FeCl_2$	0.041
$FeCl_2$ + Cytochrome $\underline{b}_5$	0.073

All tubes had final concentrations of 0.1 m$\underline{M}$ NADH, 0.01 μ$\underline{M}$ cytochrome $\underline{b}_5$

reductase, 0.14 m$\underline{M}$ methemoglobin, 25 m$\underline{M}$ bis-Tris, and 0.5 m$\underline{M}$ EDTA. When added,

the final concentration of cytochrome $\underline{b}_5$ was 0.1 μ$\underline{M}$ and the final concentration

of $FeCl_2$ was 4 μ$\underline{M}$. The pH was 7.2. Rates have been corrected for a rate of

0.005 μmole heme/hr/ml which occurs in the absence of both cytochrome $\underline{b}_5$

and $FeCl_2$. For other details see Materials and Methods.

Stimulation of reduction by 0.5 m$\underline{M}$ EDTA plus 4 μ$\underline{M}$ $FeCl_2$ in the system

reconstituted from purified reductase, cytochrome $\underline{b}_5$, and methemoglobin is

shown in Table 1. If neither iron-EDTA nor cytochrome $\underline{b}_5$ is present, the catalysis

of methemoglobin reduction by reductase is very slow (0.005 μmole heme/hr/ml).

This rate can be increased by the addition of either iron-EDTA or cytochrome $\underline{b}_5$,

and the rates with these two electron transfer agents are additive. Other

concentrations of reductase and Fe-EDTA also show additive stimulations (data

not shown).

The stimulation of methemoglobin reduction apparently results from the

action of an iron-EDTA complex which functions by transferring electrons from

the reductase to methemoglobin. We have obtained direct evidence for the reduc-

tion of methemoglobin by Fe^{+2}-EDTA. When $FeCl_2$ (2 m$\underline{M}$ final concentration) was

added to an anaerobic solution containing 0.5 m$\underline{M}$ EDTA and 0.023 m$\underline{M}$ methemoglobin,

the conversion of methemoglobin to deoxyhemoglobin was complete within the 10

seconds required to make the addition and record a visible spectrum. This

agrees with the data of Mauk and Gray (21) who have shown that Fe^{+2}-EDTA

reduces methemoglobin with a second-order rate constant of $k = 29.0 \underline{M}^{-1} s^{-1}$

$(25°, \text{pH } 7.0, \mu = 0.2 \underline{M})$.

These data suggest that iron-EDTA functions in the reconstituted methemo-

globin reduction system by the following scheme:
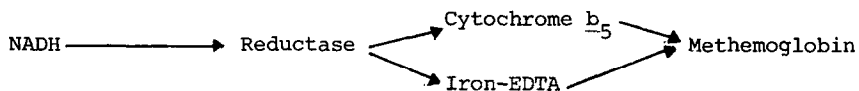
This scheme is supported by the finding that Fe^{+3}-EDTA serves as a good electron

acceptor for protease-solubilized, microsomal cytochrome $\underline{b}_5$ reductase (18).

Thus the EDTA stimulation seen in hemolysates appears to result from the

formation of EDTA complex with iron present in hemolysates, and the subse-

quent transfer of electrons from reductase to methemoglobin by this iron-

EDTA complex.

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