Vol. 198, No. 1, 1994
January 14, 1994
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 127-131

FERRIC REDUCTASES IN *ESCHERICHIA COLI* : THE CONTRIBUTION OF THE HAEMOGLOBIN-LIKE PROTEIN

Michel ESCHENBRENNER, Jacques COVES and Marc FONTECAVE*

Laboratoire d'Etudes Dynamiques et Structurales de la Sélectivité, Unité de Recherche Associée au Centre National de la Recherche Scientifique n° 332, Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 9, France

Received November 22, 1993

SUMMARY: The haemoglobin-like protein (HMP) of *E. coli* previously isolated as a dihydropteridine reductase was shown to be also a ferric citrate reductase. We demonstrate that, in fact, HMP is a flavin reductase and that its ferric reductase activity is a result of its ability to reduce free flavins. However, when compared to the two main ferric/flavin reductases of *E. coli*, *i.e.*, the NAD(P)H : flavin oxidoreductase and the sulfite reductase, one can conclude that the contribution of HMP to iron reduction is negligible.

There is now accumulating evidence that microorganisms such as *E. coli* contain a variety of ferric reductase activities involved in the complex process of iron uptake and mobilization. Many bacteria, including *E. coli*, achieve the solubilization of external Fe(III) by secreting low molecular mass chelators named siderophores. Organic acids such as citrate can also serve as iron chelators [1].

Then internalization of iron involves specific receptors and transporters. Inside the cell iron may be liberated from ferrisiderophores or ferric citrate by a reaction involving a reductive step of the metal since the ligands have a much lower affinity for the ferrous ion [2].

Our efforts to understand the mechanisms of iron mobilization in *E. coli* have led us to isolate and characterize two enzymes responsible for the reduction of various ferrisiderophores and ferric citrate. We first reported that a NAD(P)H : flavin oxidoreductase (Fre), an enzyme reducing free flavins, riboflavin, FMN or FAD at the expense of NADPH or NADH, has a very strong ferric reductase activity [3, 4]. This has been confirmed by others [5, 6].

* To whom correspondence should be addressed. Fax: 33 76 51 43 82.

Abbreviations: HMP, haemoglobin-like protein; Fre, NAD(P)H:flavin oxidoreductase; SiR, sulfite reductase, DHPR, dihydropteridine reductase.

0006-291X/94 $5.00

The flavin reductase of *E. coli* is the product of the *fre* gene. This gene has been cloned and sequenced [7]. The ferric reductase activity of soluble extracts from an *E. coli* mutant strain, named LS1312, lacking an active *fre* gene, is only 15-20 % of that of the corresponding wild type strain. From the mutant, we isolated a second ferric reductase and identified it as the sulfite reductase (SiR) [8]. Also in this case, iron reduction absolutely required the presence of free flavins, in agreement with the flavin reductase activity of the sulfite reductase recently reported [9].

Furthermore, Andrews *et al.* [5] reported the existence of a third ferric reductase activity in *E. coli*. They purified the enzyme and identified it as the haemoglobin-like protein (HMP). This protein is a flavohaemoprotein previously described as a dihydropteridine reductase (DHPR) for which a physiological function remains uncertain [10].

In this paper, we report that the ferric reductase activity of HMP also depends on the presence of free flavins and is thus due to its flavin reductase activity. Moreover we demonstrate that this ferric reductase activity is extremely low when compared to that of Fre or SiR and cannot contribute significantly to iron reduction in *E. coli*.

EXPERIMENTAL PROCEDURES

Materials: Ferric citrate was prepared as previously described [4]. Ferrozine, flavins, NADPH and NADH were purchased from Sigma. Sephacryl S-200, DEAE-Sepharose CL6B and MonoQ HR5/5 were from Pharmacia. NAD(P)H:flavin oxidoreductase was obtained in a pure form as previously described [4].

Strains and growth conditions: *E. coli* LS1312 was available in the laboratory. *E. coli* RSC521 (RSC49/pPL341) that overproduces HMP [10] was kindly provided by Dr N. Dixon (The Australian National University, Canberra, Australia). Liquid culture was grown at 37°C in Luria-Bertani medium supplemented with 0.2% glucose and ampicillin or kanamycin (50 µg/ml) respectively for RSC521 or LS1312. Growth was monitored by following absorbance at 600 nm. Cells were harvested at the end of the exponential phase by centrifugation.

Purification of HMP: Extraction of collected bacteria (9.6 g) was performed by lysozyme digestion of the membrane and freeze-thawing rupture as described in ref.[3] The purification was carried out as described by Ioannidis *et al.*[11] However, an extra purification step was included after the gel filtration to improve the purity of HMP. The pooled fractions collected from S-200 column were loaded onto a MonoQ HR5/5 (1 ml) column previously equilibrated with 50 mM Tris-Cl, pH 7.5 (buffer A). Protein was eluted at a flow rate of 1 ml/min with a linear NaCl gradient in buffer A from 0 to 0.5 M NaCl during 30 min monitored by a fast protein liquid chromatography apparatus (Pharmacia). Fractions exhibiting a peak at 410 nm were eluted with 0.18 M NaCl and pooled. The enzyme solution was desalted and concentrated by ultradialysis against buffer A supplemented by 20% glycerol.

Assays: Flavin and ferric citrate reductase activities were assayed as previously described [4] with 0.25 mM NAD(P)H and either 25 or 110 µM FMN or riboflavin. One unit of activity is defined as the amount catalyzing the oxidation of 1 nmol NAD(P)H or the reduction of 1 nmol iron per minute. Specific activity is defined as units per mg protein.

Protein concentration was determined by the method of Bradford using bovine serum albumin as standard [12].

RESULTS

From the *E. coli* strain RSC521 that overproduces HMP, we used the expeditious method described by Ioannidis *et al* [11] to get a pure enzyme after three chromatographic steps. The two first steps were an anion exchange chromatography on DEAE-sepharose CL6B and a gel filtration on Sephacryl S-200 as previously reported by Ioannidis *et al* [11]. In agreement with this report, it appeared from SDS-PAGE that the enzyme was not completely pure. Thus, purification was achieved by adding a fractionation on a MonoQ anion exchange column during which HMP eluted with 180 mM NaCl. At this stage, by SDS-PAGE analysis, only one band appeared corresponding to 44 KDa, the molecular mass of HMP. The pure protein displayed a characteristic spectrum due to the presence of FAD and heme. In this protocol, ammonium sulfate precipitations were avoided because they had been demonstrated to be harmful for the DHPR activity of HMP [10, 11].

Although the detection of HMP in chromatographic fractions of the overproducer RSC521 could be made by UV-visible spectroscopy, we also assayed the fractions from their ability to reduce ferric citrate in the presence of 25 µM FMN (Table 1). Under these conditions, HMP was purified about 22-fold, judged from the ferric reductase assay, with a final specific activity of 163. This is the first quantitative evaluation of the ferric citrate reductase activity of HMP since Ioannidis *et al* [11] did not report any enzyme activity and since Andrews *et al* [5] detected ferric reduction mainly qualitatively in native gels using an activity stain. In the last case, the purification of HMP required seven steps, including ammonium sulfate precipitations and no yield was reported.

Table 1: Purification of HMP followed by its ferric reductase activity

		Ferric reductase activity	
	Protein (mg)	Units	Specific activity
Extract RSC 521	380	2,770	7.3
DEAE Sepharose CL6B	63	945	15
S-200	3.7	174	47
MonoQ[a]	0.46	75	163

[a] Only one half of the material from the S-200 step was purified on MonoQ.

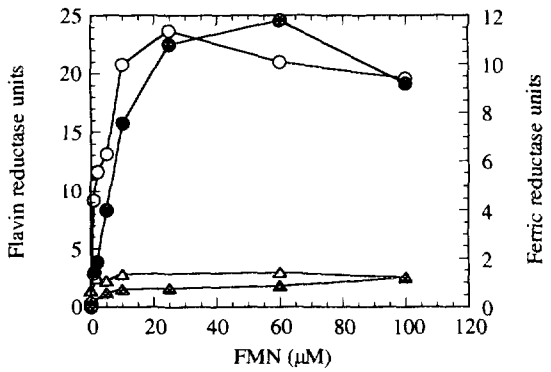

Figure 1. Comparison of flavin and ferric reductase activities from Fre and HMP. Flavin reductase (○,Δ) and ferric citrate reductase (●,▲) activities were conducted as described under Experimental Procedures with increasing amounts of FMN in the presence of either 0.67 μg Fre (○,●) or 16 μg of HMP (Δ,▲).

During ferric citrate reduction by pure HMP, NADH and NADPH could both serve as electron sources with NADH being slightly more efficient than NADPH. Moreover, rates of ferric citrate reduction were ten-fold increased by addition of free flavins. For example, with 0.25 mM NADH, 16 μg of pure HMP catalyzed the reduction of 0.26 nmol iron per minute in the absence of free flavins and of 2.62 nmol iron per minute in the presence of 25 μM FMN.

We thus verified that HMP had a flavin reductase activity. HMP was found to catalyze the oxidation of NADH or NADPH by riboflavin or FMN under aerobic conditions. In the presence of 110 μM FMN and 0.25 mM NADH a specific activity of 156 was measured (Fig. 1). No significant oxidation of NADH could be observed in the absence of free flavins.

In figure 1, we compared both ferric citrate reductase and FMN reductase activities of HMP to those of Fre. It appears clearly that for both reactions HMP activity was only about 0.5-1% of that of Fre, since assays were carried out with 16 μg of HMP and 0.67 μg of Fre. The specific ferric citrate reductase activity of Fre was 16 μmol reduced iron per minute and per mg protein.

DISCUSSION

The haemoglobin-like protein (HMP) from *E. coli* has been recently shown to display a ferric citrate reductase activity [5]. In fact, we show now that its ability to reduce iron complexes such as ferric citrate is due to its flavin reductase activity. HMP is able to catalyze the transfer of electrons from NADPH or NADH to riboflavin or FMN. It is, in the second step, the enzymatically reduced flavins which in turn reduce Fe(III) into Fe(II). Accordingly, almost no reduction of iron could be observed in the absence of free flavins. It is remarkable that the two first ferric reductases of *E. coli* previously reported, Fre and the sulfite reductase, were also flavin reductases and

absolutely required the presence of free flavins [4, 7, 8]. This gives extra support to our hypothesis of the importance of free flavins for iron reduction in general and that in *E. coli* as well as in most microorganisms ferric reductases are flavin reductases [13].

Since several enzymes are now suspected to be involved in the reduction of iron in *E. coli*, it is important to precise their relative contribution to such a reaction, at least from the quantitative evaluation of their Fe-reducing power *in vitro*. The NAD(P)H:flavin oxidoreductase has a specific ferric citrate activity of 16. 000, a value about 10-fold higher than that of the sulfite reductase [8]. Moreover, the affinity of the sulfite reductase for free flavins is much lower than that of Fre, with Km values of 15-30 µM compared to 1-2 µM respectively [3, 9]. Even though there are still uncertainties on the intracellular concentration of free flavins, one can assume that not more than a few µM free flavin is available. Consequently, one may suggest that the contribution of Fre to iron reduction will be stronger than that of the sulfite reductase. It is noteworthy that ferric reductase of a Fre-deficient *E. coli* strain was only 15-20% of the activity of the corresponding wild-type strain [4]. Furthermore under identical assay conditions, the ferric citrate reductase activity of HMP was only 0.16 µmol iron reduced per minute and per mg protein, *i.e.* 1% of the Fre ferric reductase activity.

Even though these calculations have not taken all the parameters into account, for example the relative intracellular concentration of the various Fe-reducing enzymes, we would like to suggest that the contribution of HMP to iron reduction in *E. coli* is in all probability negligible and that Fre is the major ferric reductase.

REFERENCES

1. Crichton, R.R. (1991) in Inorganic biochemistry of iron metabolism (Burgess, J., ed.) Ellis Horwood, New York, London, Toronto, Sydney, Tokyo, Singapore.
2. Neilands, J.B. (1981) Ann. Rev. Biochem. 50, 715-731.
3. Fontecave, M., Eliasson, R. and Reichard, P. (1987) J. Biol. Chem. 262, 12325-12331.
4. Covès, J. and Fontecave, M. (1993) Eur. J. Biochem. 211, 635-641.
5. Andrews, S.C., Shipley, D., Keen, J.N., Findlay, J.B.C., Harrison, P.M. and Guest, J.R. (1992) FEBS Lett. 302, 247-252.
6. Fischer, E., Strehlow, B., Hartz, D. and Braun, V. (1990) Arch. Microbiol. 153, 329-336.
7. Spyrou, G., Haggard-Ljungquist, E., Krook, M., Jörnvall, H., Nilsson, E. and Reichard, P. (1991) J. Bacteriol. 173, 3673-3679.
8. Covès, J., Eschenbrenner, M. and Fontecave, M. (1993) Biochem. Biophys. Res. Comm. 192, 1403-1408.
9. Covès, J., Nivière, V., Eschenbrenner, M. and Fontecave, M. (1993) J. Biol. Chem. 268, 18604-18609.
10. Vasudevan, S.G., Armarego, W.L.F., Shaw, D.C., Lilley, P.E., Dixon, N.E. and Poole, R.K. (1991) Mol. Gen. Genet. 226, 49-58.
11. Ioannidis, N., Cooper, C.E. and Poole, R.K. (1992) Biochem. J. 288, 649-655.
12. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
13. Fontecave, M., Covès, J. and Pierre, J.L. (1994) BioMetals (in press).