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Cyclic Voltammetry of Human Dimeric Cystathionine β-synthase

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Cystathionine β-synthase (CBS) catalyzes the pyridoxal phosphate-dependent condensation of serine and homocysteine into cystathionine and participates in the transsulfuration pathway which converts methionine into cysteine. CBS is unique in having a heme cofactor of unknown function with axial cysteine and histidine ligands. Since the heme does not participate in the enzymatic mechanism, it could be involved in the redox regulation of the enzyme. In this study, we have assessed the redox behavior of CBS by direct electrochemistry and we have estimated its reduction potential by cyclic voltammetry. Human dimeric CBS, a variant lacking the C-terminal domain, showed a quasi-reversible response on a platinum electrode with anodic and cathodic peaks at –0.325 V and –0.454 V vs NHE, respectively, yielding an estimated formal reduction potential of –0.158 V vs NHE at pH 6.46. Similar electrochemical profiles were obtained with CBS that had been treated with N-ethylmaleimide to block the thiols. The results obtained are interpreted in terms of the known structure of CBS and suggest that the heme could be in the reduced state *in vivo*.

Keywords: Cystathionine β-synthase, Cyclic voltammetry

1. INTRODUCTION

Cystathionine β-synthase (CBS[1], EC 4.2.1.22) is a key enzyme of homocysteine metabolism in mammals that catalyzes the condensation of homocysteine and serine to form cystathionine in the first

[1] The abbreviations used are: CBS, cystathionine β-synthase; PLP, pyridoxal 5´-phosphate; AdoMet, S-adenosyl-L-methionine; NHE, normal hydrogen electrode; NEM, N-ethylmaleimide.

reaction of the transsulfuration pathway that leads to the formation of cysteine. Mutations in CBS are the single most common cause of severe hyperhomocysteinemia [1] Increased levels of homocysteine in plasma constitute an independent risk factor for cardiovascular diseases].

Human CBS is a cytosolic homotetramer with a subunit molecular weight of ~63 kDa. Each polypeptide chain has a modular organization that comprises an N-terminal region that binds heme, followed by a catalytic domain which binds pyridoxal 5´-phosphate (PLP) and a C-terminal regulatory domain that binds the allosteric activator S-adenosyl-L-methionine (AdoMet) [3, 4]. CBS also has a disulfide oxidoreductase motif which contains the sequence CPGC and shares a common folding pattern with *E. coli* glutaredoxin [5, 6]. Limited proteolysis results in the separation of the C-terminal AdoMet regulatory domain leading to a more active enzyme and a change in its oligomeric state from a tetramer to a dimer of 45 kDa subunits (4). The crystal structure of human dimeric CBS has already been reported [5, 6].

CBS is the only known PLP-dependent enzyme that contains heme. The heme in CBS is an unusual iron protoporphyrin IX that contains an axial cysteine ligand (Cys52), like other heme-thiolate proteins such as cytochrome P450, chloroperoxidase, and nitric oxide synthases. In addition, CBS has, as sixth ligand, a histidine residue (His65) [5]. The ferric enzyme exhibits a heme Soret peak at 428 nm which shifts to 450 nm in the ferrous reduced state [7]. The heme in both the ferric and ferrous states is six-coordinated and low spin. The function of the heme in CBS remains unknown. A catalytic role in the PLP-mediated enzymatic mechanism was excluded by spectroscopic studies and is also consistent with its absence from the highly homologous yeast enzyme, which catalyzes the same reaction but does not contain heme [8, 9]. An alternative regulatory role has been considered for this cofactor. In this sense, reduction of the heme to the ferrous state decreased the enzyme activity by ~50 %, suggesting that the heme could act as a redox sensor [7].

In order to understand the role of this very unusual cofactor and study its possible roles *in vivo*, it is critical to evaluate its redox properties. In this work, we performed cyclic voltammetry to estimate the reduction potential of the heme in human dimeric CBS and analyzed the results in light of the known protein structure.

2. EXPERIMENTAL PART

Enzyme purification. The truncated human CBS lacking 143 amino acids at the C-terminus was purified from a recombinant expression system (pGEX4T1/hCBS) that produces a fusion protein with glutathione *S*-transferase [10]. The cells were cultured and the protein was purified as described previously [7]. Enzyme activity was measured using the ninhydrin method [11] and protein concentration was determined by the Bradford method, using bovine serum albumin as standard [12]. The enzyme had a specific activity of ~300 μmol h^{-1} mg^{-1} at 37 ºC, in accordance with reported values[7, 13]. Thiols were quantified using 5,5´-dithiobis-(2-nitrobenzoic acid) (DTNB) (ε_{412} = 13.6 mM^{-1} cm^{-1}) [14], after ultrafiltration with Ultrafree 0.5 centrifugal filter devices (Millipore) to remove interference from the cofactors at 412 nm. In our conditions we obtained one thiol per CBS monomer.

Cyclic voltammetry. Cyclic voltammograms were recorded at room temperature in a 1 mL standard three-electrode glass cell using a BAS EC Epsilon potentiostat /galvanostat (Bioanalytical Systems Inc.). A platinum working electrode was used, which yielded better electrochemical responses than gold and glassy carbon working electrodes. The counter electrode was a large platinum wire and the reference was a saturated Ag/AgCl electrode, which was routinely patronized. The potential values are reported vs the normal hydrogen electrode (NHE), obtained by adding 0.200 V to the measured potential vs the reference Ag/AgCl electrode. Human dimeric CBS was used at a final concentration of 10 μM in 0.1 M potassium perchlorate as supporting electrolyte. The final pH of the mixtures was always controlled before and after measurements and was ~6.5. To account for the effect of the protein thiols, CBS was incubated with an excess of *N*-ethylmaleimide (NEM, 1 mM), for 30 min at 25 °C, followed by gel filtration on microBio-Spin chromatography columns (Bio-Rad) equilibrated with potassium perchlorate (0.1 M). NEM treatment left no residual thiols and in this case the final pH of the mixtures was 6.98. Voltammograms were taken in solutions that had been carefully deoxygenated by bubbling with 99.998 % pure nitrogen over the sample and through the cell for 3 min prior to the experiment. Millipore–MilliQ* plus water of 18.2 MΩ cm resistivity was used for the electrolyte preparation. Data are the mean of replicates from a representative experiment that differed in less than 5 %.

3. RESULTS

Cyclic voltammetry has been widely employed to study the electrochemical properties of hemeproteins. In the case of CBS, initial experiments using the full length enzyme were complicated due to protein aggregation on the electrode surface. Thus, we decided to study the dimeric CBS. The cyclic voltammogram of dimeric CBS at a scan rate of 0.1 V s^{-1}, as depicted in Fig. 1 (panel A), exhibited anodic and cathodic peaks centered at −0.325 V and −0.454 V vs NHE, respectively, with a peak separation of ca. 0.129 V, which indicates a quasi-reversible (considering a single electron transfer with a high degree of reversibility). Since the peak separation was higher than 0.059 V, the formal reduction potential could not be calculated as the average of the cathodic and anodic peaks. Instead, with the assumption that the concentration of the reduced species was constant, it was possible to estimate the reduction potential using a pseudo-nernstian equation:

$$E = E' - \frac{2.3RT}{nF} \log \frac{[oxidized]}{[reduced]} \qquad (1)$$

where R is the gas constant, F is Faraday's constant, T is the absolute temperature and n is the number of electrons transferred per mole of analyte. The quotient (oxidized)/(reduced) can be evaluated either by the surface concentrations of the oxidized and reduced enzyme or by the $(I_p - I)/I$ ratio, being I_p and I the maximum current intensity and that obtained in the nearness of the electrode surface [25].

For an oxidized enzyme concentration of 10 μM, the formal reduction potential of dimeric CBS was estimated as −0.158 V vs NHE at pH 6.46. As expected, no redox wave was observed in the

absence of the enzyme using the background electrolyte potassium perchlorate (0.1 M). Panel B shows the cyclic voltammogram of dimeric CBS at a scan rate of 1 V s^{-1}. The electrode response was dominated by a similar quasi-reversible redox couple with essentially the same peaks as obtained previously at a lower scan rate, but in addition another couple was observed at –0.030 and –0.229 V vs NHE for the anodic and cathodic peaks, respectively. In the case of an electrochemical system obeying a mixed adsorption and diffusion control kinetics, a modification of the Randles-Sevčik equation arises [26];

$$I_p = 2.6910^5 n^{3/2} A D^{1/2} C^o v^{1/2} + Bkv \quad (2)$$

being, A the electrode area, D the diffusion coefficient of the oxidized soluble species, C^o the bulk concentration for the oxidized species, v the potential scan rate, B a numerical constant and k a physicochemical parameter representing the adsorption isotherm obeyed by the enzyme on the platinum electrode surface. In our case it was possible to check that the contribution of the first term, *i.e.* the diffusional term, is ca. 70 % of the total current. The adsorption of enzymes containing heme groups has been found before by Roscoe *et al.* [27].

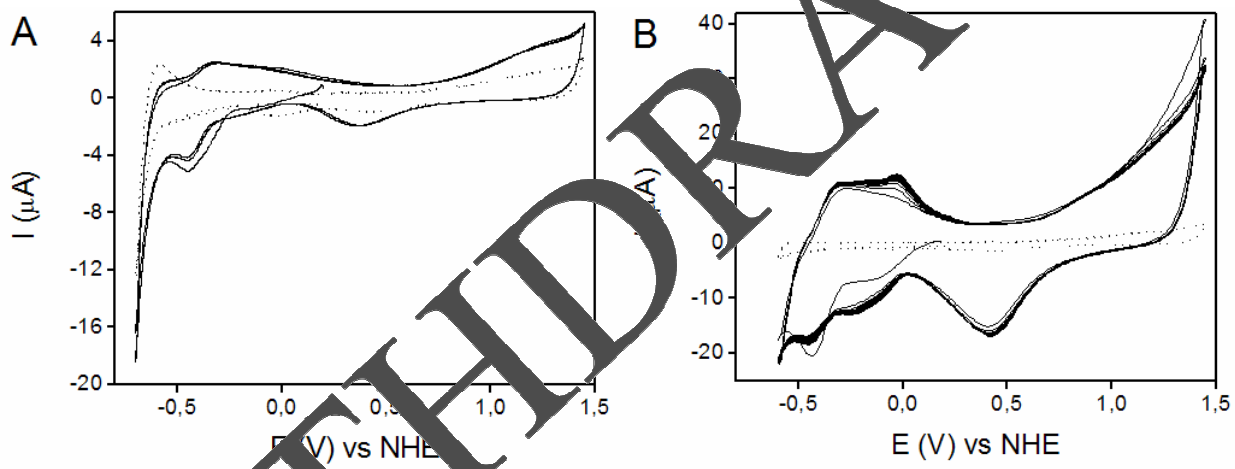

Figure 1. Cyclic voltammetry of dimeric CBS recorded in deoxygenated potassium perchlorate solution (0.1 M), final pH 6.4, at a scan rate of 0.1 V s^{-1} (panel A), or 1 V s^{-1} (panel B) in the absence (dashed line) or in the presence of 10 μM CBS (solid line).

On the other hand, the dependence of the cyclic voltammogram on the potential scan rate shows, according to [28], a first quasi-reversible electrochemical step, followed by an irreversible chemical pathway with a final quasi-reversible electrochemical path ($E_qC_iE_q$ mechanism). The difference in the cyclic voltammetric runs between 0.01 and 1.0 V s^{-1} shows that the second electron transfer can be evidenced only at sweep rates larger than 0.05 V s^{-1}, so the irreversible chemical path is the *rds* of the process.

To determine whether thiols in CBS could account for the redox waves observed, the thiol groups were alkylated with NEM. Similar voltammograms were obtained for thiol-blocked CBS at

both scan rates of 0.1 and 1 V s^{-1} (data not shown), which is consistent with the fact that the redox response observed originated on the heme.

4. DISCUSSION

The cyclic voltammograms indicated that the heme iron in CBS undergoes quasi-reversible reduction to the ferrous state with anodic and cathodic peaks at –0.325 and –0.454 V respectively, yielding an estimated reduction potential of –0.158 V vs NHE. This value is in the range of other heme-thiolate proteins which also have relatively negative reduction potentials, such as chloroperoxidase (–0.140 V, [15] cytochrome P450 (–0.170 to –0.360 V [16] and nitric oxide synthase (–0.347 to –0.239 V, [17], in agreement with the strong electron-donating nature of the Cys52 thiolate.

Since the direct electrochemical reactions of proteins at metal electrodes are often irreversible and sometimes undetectable, the fact that CBS yielded a quasi-reversible response through cyclic voltammetry in the absence of mediators is remarkable and indicates that the heme is relatively accesible and solvent exposed, on line with the structural data shown in Fig. 2 (5, 6). Moreover, as it was found before for other heme-containing enzymes [27] the surface charge density accumulated on the electrode was attributed to a denaturing of the protein at the surface via carboxylate groups adsorption accompanied by electron transfer at quasi-reversible conditions. In addition, the fact that a second electrochemical contribution was obtained at higher scan rates suggests a complex mechanism as explained above for the enzyme ($E_qC_iE_q$ mechanism).

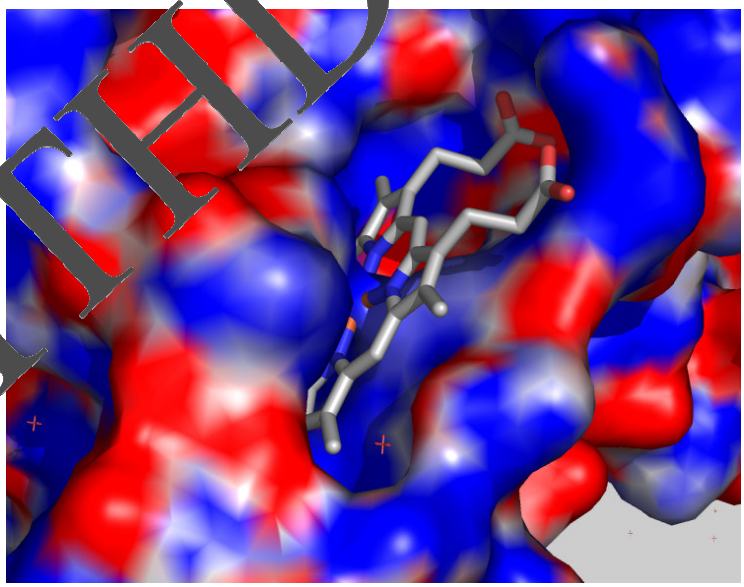

Figure 2. Three-dimensional structure of the heme in CBS showing the electrostatic surface. Electropositive and electronegative densities are depicted in blue and red, respectively. CBS structural data was obtained from the structure data bank of PubMed, accession code 1JBQ [5]. The figure was generated using PyMOL Molecular Graphics System [23], using Adaptive Poisson-Boltzmann Solver (APBS) for electrostatic calculations [24].

The redox potential of hemeproteins is influenced by several factors including the identities of the amino acids ligating the heme, the hydrophobicity of the heme binding site, heme exposure and solvent accesibility, as well as hydrogen bonding interactions within the heme cavity and electrostatic interactions with charged residues.

The effect of the ligands can be interpreted in terms of the "push-pull" concept [18]. In CBS, the axial Cys52 thiolate is engaged in polar interactions with Arg266 and the main chain nitrogen of Trp54 [5]. These interactions, by diminishing the electron donation to the metal center, would tend to increase the redox potential. Analogously, the His65 axial ligand in CBS is relatively solvent accesible and, in constrast to the globins and peroxidases, lacks any hydrogen bonding partners [5]. This would tend to decrease the imidazolate character of the ligand and increase the reduction potential.

As for the effects of the heme environment, it has been proposed that the reduction potential decreases as the polarity of the heme increases because this tends to stabilize a more highly charged oxidized state [19-21]. In contrast to other proteins where the heme is completely buried in an internal cavity of the protein, the heme in CBS is relatively surface exposed, which would tend to lower the reduction potential. However, the heme environment in CBS appears to be relatively electropositive. This can be explained because there is a higher number of positively charged residues within close range of the heme than negatively charged ones and the heme carboxylates are shielded by interactions with Arg51 and Arg224 and with the solvent. As highlighted in Fig. 2, this electropositive environment would stabilize the reduced state and increase the reduction potential.

On the basis of the data reported here, it is proposed that the heme in its particular coordination environment in CBS has a reduction potential high enough to allow the protein to be reduced *in vivo*. Although this interpretation needs to be taken cautiously since the heme in CBS has been reported to reoxidize spontaneously in a pH-dependent step [22], our results are consistent with the proposed regulatory role of the reduced state of the heme and will aid in the understanding of this unusual cofactor.

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