ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 19–20 (2002) 437–441

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Chemical modification of heme group improves hemoglobin affinity for hydrophobic substrates in organic media

Eduardo Torres [a,*], Alejandro Baeza [b], Rafael Vazquez-Duhalt [c]

[a] *Depto. de Biotecnología, Instituto Mexicano del Petróleo, Programa de Biotecnología, Eje Central Lázaro Cárdenas No. 152, Delegación Gustavo A. Madero 07730, Mexico D.F., Mexico*

[b] *Facultad de Quimica, Universidad Nacional Autonoma de Mexico. Ciudad Universitaria. Mexico D.F., Mexico*

[c] *Instituto de Biotecnologia, Universidad Nacional Autonoma de Mexico, Apartado Postal 510-3, Cuernavaca, Morelos 62250, Mexico*

Received 28 September 2001; received in revised form 29 April 2002; accepted 13 May 2002

Abstract

Carboxylic groups of heme prosthetic group from hemoglobin were modified with *p*-nitrophenol and *p*-aminophenol, using a carbodiimide chemistry, to change its electron character and to increase its hydrophobicity. The modification of heme group included the extraction of heme group by the method of acid acetone, the chemical modification of protoporphyrin IX and the reincorporation of modified heme group in apohemoglobin. The effect of the chemical modification on substrate affinity and catalytic activity were studied. Dissociation constants in aqueous media using different substrates showed that chemical modification of hemoglobin active-site improved the substrate affinity up to 30 times. In addition, the chemical modification slightly increased the solvent concentration at which hemoglobin was catalytically active. This biocatalytic behavior could be attributed to the hydrophobicity increase of active site. On the other hand, the chemical modification of the heme prosthetic group altered its electron balance affecting the specific activity of hemoglobin.

Keywords: Chemical modification; Heme; Hemoglobin; Hydrophobicity; Organic solvents

1. Introduction

Organic solvents as reaction media have significantly increased the range and efficiency of practical applications of enzymes [3]. In these systems, hemoproteins are able to catalyze the oxidation of some toxic compounds like polycyclic aromatic hydrocarbons, dyes and phenols. However, the oxidation of these hydrophobic compounds by hemoproteins is highly limited by the substrate partition between the protein active site and the bulk solvent. As the concentration of organic solvent increases the interaction between the hydrophobic substrate and the protein active site decreases, and then the biocatalytic activity is reduced. In a previous work we proposed a thermodynamic model based on this substrate partition able to predict the biocatalytic behavior of lignin peroxidase and cytochrome c in systems containing different water-miscible organic solvents [13]. The model also predicts limitations for enzymatic reactions on hydrophobic compounds with three other peroxidases (lactoperoxidase, horseradish peroxidase, and chloroperoxidase) in a wide range of solvent hydrophobicities [12].

* Corresponding author. Tel.: +52-55-3003-7238/3003-7017; fax: +52-55-5368-1400/3003-7765.
E-mail address: etorres@imp.mx (E. Torres).

1381-1177/02/$ – see front matter
PII: S1381-1177(02)00196-0

Chemical modification of heme have been reported as a technique to overcome the problem of substrate partition. Modi et al. [5] reported an increase of substrate affinity of cytochrome P450 when the heme was replaced with a heme dimethyl ester. An increment of both, surface and active site hydrophobicities, through covalent coupling of poly(ethylen)glycol in surface free amino groups, and the methyl esterification of heme propionates, increased the specific activity and the substrate range of cytochrome c for the oxidation of polycyclic aromatic hydrocarbons [11]. The catalytic enhancement was attributed to an affinity increase for hydrophobic substrates in the modified protein.

In this work, the hydrophobicity and the electron character of heme prosthetic group of hemoglobin were altered by chemical methods with the aim to improve the catalytic activity. The modification of hemoglobin included the extraction of heme group, the chemical modification of protoporphyrin IX, and the reincorporation of modified heme group in the apohemoglobin. The effect of this modification on the substrate affinity and the catalytic activity for hydrophobic compounds were evaluated.

2. Experimental

2.1. Chemicals

Human hemoglobin, oxalic acid, *p*-nitrophenyl alcohol, *p*-aminophenyl alcohol, hemin chloride, ethyl-3-(-dimethylaminopropyl) carbodiimide, phenol, resorcinol and guaiacol were obtained from Sigma Chemical. Buffer salts and hydrogen peroxide were purchased from J.T. Baker.

2.2. Heme extraction from human hemoglobin

The heme from hemoglobin was extracted using the acid acetone method according Ascoli et al. [1]. Ten miligrams of protein were added dropwise to cooled (−20 °C) acid solution of acetone. After 10 min, the solution was centrifuged at 10 000 g for 10 min at −10 °C. The apohemoglobin obtained was redissolved in water and exhaustively dialyzed in 10 mM phosphate buffer before heme reincorporation experiments.

2.3. Preparation of heme derivatives

Heme carboxylic groups were modified with *p*-nitrophenol and *p*-aminophenol using a carbodiimide chemistry [9,10]. Five milligrams of hemin chloride were mixed with 10 molar excess of either *p*-nitrophenyl alcohol or *p*-aminophenyl alcohol in 10 ml dimethylformamide. Ethyl-3-(-dimethylaminopropyl) carbodiimide (10 molar excess) was added and the solution was maintained under constant stirring at room temperature (25 °C). After 24 h, the solution was acidified with HCl until heme precipitation. The precipitate was washed with water, dried and redissolved in a minimum volume of dichloromethane–methanol (10:1 v/v). The modified heme was separated from unmodified heme by chromatography on a silica gel column. The compounds were eluted using a dichloromethane–methanol phase (5:1 v/v). Unmodified heme was collected, according to heme absorption spectra, obtaining a single product.

2.4. Heme reincorporation

Holoprotein was regenerated incubating apohemoglobin with a 5 molar excess of heme chloride (H-hemoglobin), *p*-nitrobenzene-heme (PNP-hemoglobin) or *p*-aminobenzene-heme (PAP-hemoglobin) in 10 mM phosphate buffer containing 5% of dimethylformamide. The solution was incubated for 12 h at 4 °C under constant stirring. The resulting solution was dialyzed against 60 mM buffer phosphate to eliminate the solvent and separate the denatured protein. The excess of the unbound heme was removed by two chromatography steps. First, the dialyzed solution was concentrated in an ultracentrifugation cell (10 000 MW) to a minimum volume, and chromatographied on a Sephadex G25 column. Then, the enzyme-containing solution was chromatographied on an ionic-exchange column (CM cellulose). Finally, the eluted protein was dialyzed against 60 mM phosphate buffer at 4 °C.

2.5. Substrate binding studies

Dissociation constants were determined as previously reported for human hemoglobin and cytochrome P450 [5,14], as follows. The measurements were carried out in 1 ml cell containing a protein solution in

60 mM phosphate buffer, pH 6, and under nitrogen atmosphere. Microliter amounts of concentrated solutions of phenols were added to the sample cell and an equivalent volume of buffer to the enzyme solution in the reference cell After reaching the equilibrium, the difference spectra was recorded. The dissociation constants were calculated from the following linear equation:

$$\frac{1}{\Delta A} = \left(\frac{k_d}{\Delta A_s}\right)\left(\frac{1}{S_0}\right) + \frac{1}{\Delta A_s}$$

where ΔA and ΔA_s are the absorbance changes at 405 nm at a given initial (S_0) and saturating substrate concentrations respectively, and k_d is the dissociation constant of the enzyme–substrate complex.

2.6. Determination of heme-protein relation in reconstituted hemoglobin

Protein concentrations were estimated by protein measurements with the Bio-Rad reagent procedure. Heme concentration was determined as iron in heme according to Rice-Evans et al., [8] procedure using a standard solution of hemoglobin for the calibration curve.

2.7. Reactions conditions

Hemoglobin activity in 15% acetonitrile was determined in 1 ml reaction mixture containing 20 μM thianthrene, 10 mM H_2O_2 in a 60 mM phosphate buffer, pH 6. The reaction progress was followed spectrophotometrically at 254 nm during 2 min. Specific activities were calculated by using an extinction coefficient of thianthrene of 35 mM^{-1} cm^{-1} [13].

2.8. Activity in different acetonitrile–water mixtures

Biocatalytic activities of hemoglobin were estimated in 1-ml reaction mixture containing 20 μM thianthrene, 10 mM H_2O_2 in a 60 mM phosphate buffer, pH 6 in different acetonitrile concentrations up to a concentration where no reaction was detected. The reaction progress was followed spectrophotometrically at 254 nm during 2 min. The activity was calculated as a percent of the highest activity found.

3. Results and discussion

Hemoglobin is able to catalyze the oxidation of some hydrophobic substrates such as polycyclic aromatic hydrocarbon (PAHs) in organic systems [7]. A double chemical modification of hemoglobin increased the specific activity against PAHs [14]. The higher activity was reported to be a consequence of an increment of enzyme hydrophobicity. In this work, a different approach was assayed in order to alter both, the hydrophobicity and the electron affinity of heme prosthetic group from hemoglobin.

Electronic absorption spectra of modified and unmodified hemoglobin demonstrated that the active site was really modified. Control (H-hemoglobin) and unmodified hemoglobin (native hemoglobin) have a maximum absorption band at 403 nm, while both PNP-hemoglobin and PAP-hemoglobin have their maximum absorption at 410 nm (Fig. 1). Absorption spectra of PNP- and PAP-hemoglobins were indistinguishable, suggesting a similar alteration of the active site environment. Protein and heme iron determinations showed that the number of modified heme groups by protein is four, which is consistent with the unmodified hemoglobin.

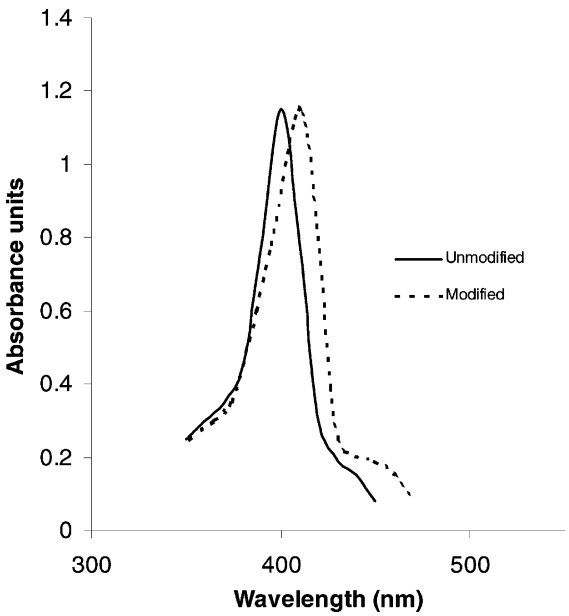

Fig. 1. Electronic absoption spectra of unmodified and modified (PNP and PAP) hemoglobin.

Table 1
Dissociation constants of unmodified and modified hemoglobin

Substrate	Water solubility (M)	k_d (mM)			
		Unmodified	PNP-hemoglobin	PAP-hemoglobin	Peg-Met-hemoglobin[a]
Resorcinol	10	221	31.0	24.3	138
Phenol	0.7	29.8	19.2	20.5	13.1
Guaiacol	0.12	45.8	1.4	1.5	3.6

[a] PEG-MET hemoglobin data from [14].

Dissociation constants were determined for phenolic substrates with different hydrophobicities (Table 1). The results show that the affinity of chemically modified hemoglobin could be improved up to 30 times, and the more hydrophobic substrate the higher substrate affinity. This affinity increase by the esterification of heme propionates was also found in the doubly modified hemoglobin [14] (Table 1). It appears that methylation of heme propionate groups removes the negatives charges from the vicinity of the substrate-binding site, increasing its hydrophobic nature, and increasing the substrate affinity.

Replacing water with an organic solvent affects considerably the binding energy, because it is more difficult to desolvate the hydrophobic substrate from organic solvent than from an aqueous media. Thus, the highest solvent hydrophobicity the weaker energy binding, and in consequence, lower enzymatic activity [4]. From the dissociation constants data it appears that in an organic media the reconstituted hemoglobins may have a higher energy binding (a more favorable substrate partition) than the unmodified hemoglobin, because their higher active-site hydrophobicity. Peroxidase activity profile in increasing concentrations of organic solvent could be used as a measure of substrate partition [12]. Fig. 2 shows the profile of catalytic activity of hemoglobin for the oxidation of thianthrene with different concentrations of acetonitrile. Chemical modification of active-site slightly increased the solvent concentration at which hemoglobin was able to perform catalysis on the hydrophobic substrate. Reconstituted

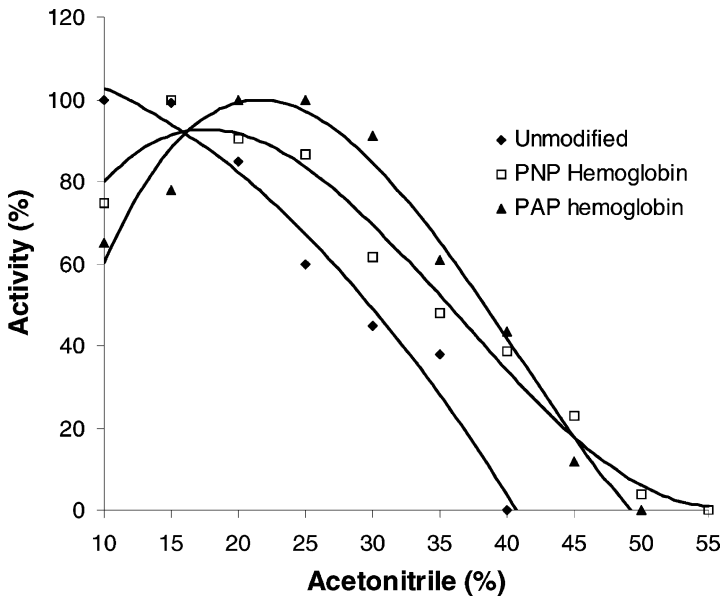

Fig. 2. Effect of organic solvent concentration on thianthrene oxidation of unmodified and reconstituted hemoglobins.

Table 2
Specific activity of unmodified and chemically modified hemoglobins for thianthrene oxidation in reaction system containing 15% acetonitrile

Hemoglobin	Specific activity (min^{-1})
Unmodified hemoglobin	12.6
PNP-hemoglobin	39.0
PAP-hemoglobin	5.7

PNP- and PAP-hemoglobins were active up to 50 y 45% acetonitrile respectively, while the unmodified hemoglobin was only active up to 35% acetonitrile. This biocatalytic behavior could be attributed to the hydrophobicity increase of active site.

Recently, horseradish peroxidase and myoglobin reconstitutions with chemical modified heme were studied, and the modification led to a higher accessibility to active site increasing their catalytic activity [2,6]. Our results shows that the heme modification can increase or decrease the specific activity (Table 2). Reconstituted PNP-hemoglobin has a higher specific activity on thianthrene in a system with 15% acetonitrile than unmodified hemoglobin, while PAP-hemoglobin showed lower activity than the unmodified hemoglobin. This biocatalytic behavior could be attributed to the electron character of the substituted group. When the carboxylic acids of heme are modified with an electron attractor, *p*-nitrophenol (PNP-hemoglobin), the redox potential of hemoglobin is expected to be increased, increasing enzyme activity. On other hand, electron density at the heme moiety is increased when the propionates from heme are esterified with *p*-aminophenol, an electron donor (PAP-hemoglobin), and the electron transfer rate is expected to decrease, diminishing the specific activity.

Thus, the results obtained indicate that it is possible to increase both affinity and catalytic activity of hemoglobin by the chemical modification of heme group.

Acknowledgements

This work has been supported by the Mexican Petroleum Institute (Project D. 00020 and grant FIES 98-110-VI).

References

[1] F. Ascoli, M.R.S. Fanell, E. Antonini, Preparation and properties of apohemoglobin and reconstituted hemoglobins, Methods Enzymol. 76 (1981) 72.
[2] T. Hayashi, Y. Hitomi, T. Ando, T. Mizutani, Y. Hisaeda, S. Kitagawa, H. Ogoshi, Peroxidase activity of myoglobin enhanced by chemical mutation of heme-propionates, J. Am. Chem. Soc. 121 (1999) 7747.
[3] Y.L. Khmelnitsky, J.O. Rich, Biocatalysis in nonaqueous solents, Curr. Opoin. Chem. Biol. 3 (1999) 47.
[4] A. Klibanov, Why are enzymes less active in organic solvents than in water? TIBTECH 15 (1997) 97.
[5] S. Modi, W.V. Primrose, L.Y. Lian, G.C.K. Roberts, Effect of replacement of ferroporphyrin IX in the haem domain of cytochrome P450BM-3 on substrate binding on catalytic activity, Biochem. J. 310 (1995) 939.
[6] E. Monzani, G. Alzuet, L. Casella, C. Redaelli, C. Bassani, A.M. Sanangelantoni, M. Gullotti, L. De Gioia, L. Santagostoni, F. Chillemi, Properties and reactivity of myoglobin reconstituted with chemically modified protohemin complexes, Biochemistry 39 (2000) 9571.
[7] M. Ortiz-Leon, L. Velasco, R. Vazquez-Duhalt, Biocatalytic oxidation of polycyclic aromatic hydrocarbons by hemoglobin and hydrogen peroxide, Biochem. Biophys. Res. Commun. 215 (1995) 968.
[8] C.A. Rice-Evans, A.T. Diplock, M.C.R. Symons, Techniques In Free Radical Research. Elsevier, Amsterdam,1991.
[9] A.D. Ryabov, V.N. Goral, L. Gorton, E. Csöregi, Electrochemically and catalycally active reconstituted horseradish peroxidase with ferrocene-modified hemin and a artificial binding site, Chem. Eur. J. 3 (1999) 961.
[10] K. Takahashi, A. Matsushima, Y. Saito, Y. Inada, Polyethylene glycol-modified hemin having peroxidase activity in organic solvents, Biochem. Biophys. Res. Commun. 138 (1986) 283.
[11] R. Tinoco, R. Vazquez-Duhalt, Chemical modification of cytochrome c improves their catalytic properties in the oxidation of polycyclic aromatic hydrocarbon, Enzyme Microb. Technol. 22 (1998) 8.
[12] E. Torres, B. Siminovich, E. Barzana, R. Vazquez-Duhalt, Thermodynamic hydrophobicity of aqueous mixtures of water-miscible organic solvents predicts peroxidase activity, J. Mol. Catal. B Enzymatic 4 (1998) 155.
[13] E. Torres, R. Tinoco, R. Vazquez-Duhalt, Solvent hydrophobicity predicts biocatalytic behaviour of lignin peroxidase and cytochrome c in aqueous solution of water-miscible organic solvents, J. Biotechnol. 49 (1996) 59.
[14] E. Torres, R. Vazquez-Duhalt, Chemical modification of hemoglobin improves biocatalytic oxidation of PAHs, Biochem. Biophys. Res. Commun. 273 (2000) 820.