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THERMODYNAMICS OF CLEANING-UP OF WASTE WATERS POLLUTED BY CHROMIUM
Using sulfate-reducing bacteria: preliminary results

B. Chardin[1], *P. Gallice*[2*], *J. C. Sari*[2] *and M. Bruschi*[1]

[1]Laboratoire de Bioénergétique et Ingénierie des Protéines, IBSM-CNRS 31 ch. Joseph Aiguier, 13009 Marseille, France
[2]Laboratoire de Chimie Générale et Physico-Chimie de l'Environnement, Faculté de Pharmacie, 27 bd. Jean Moulin, 13385 Marseille Cedex 05, France

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Abstract

The effect of Cr(VI) on *Desulfovibrio vulgaris* strain Hildenborough bioenergetic metabolism was monitored by microcalorimetry and the concomitant reduction of this metal was studied. Results showed that Cr(VI) is reduced by the bacterium and that the bacterial growth is altered, involving a strong modification of the metabolism of the bacteria. An absence of correlation between Cr(VI) reduction and cell growth is observed, suggesting that Cr(VI) does not yield energy to support anaerobic growth. The analysis of the enzymatic characteristics of Cr(VI) reduction are in progress.

Keywords: chromium reduction, metabolism, microcalorimetry, sulfate-reducing bacteria

Introduction

Chromium is one of the most widely used metals in industry and is responsible of a widespread pollution [1, 2]. The hexavalent chromium Cr(VI) is highly water-soluble and known to be carcinogenic, mutagenic and a strong oxidizing agent. Conversely, the trivalent form Cr(III) is much less toxic and tends to form insoluble and stable hydroxides [3, 4]. Because the toxicity of chromium is a function of its oxidation state and its aqueous concentration, the understanding of the reactions that control the oxidation state and aqueous concentrations is of great importance. Existing chemical reduction techniques, used in the treatment of wasted soils or waters containing Cr(VI), are often expensive and may themselves produce hazardous by-products [5]. In consequence, these techniques lead to a complementary confinement of polluted materials which does not allow their reuse. Therefore, the microbial reduction of toxic Cr(VI) to Cr(III) and its subsequent precipitation may represent a useful detoxification technique, efficient, cheaper and environmental friendly.

* Author for correspondence: E-mail: gallice@pharmacie.univ-mrs.fr

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Akadémiai Kiadó, Budapest

Microorganisms, such as *Escherichia coli*, *Bacillus subtilis*, *Enterobacter cloacae*, *Deinococcus radiodurans* or sulfate-reducing bacteria, can reduce Cr(VI) [6–8]. Among them, sulfate-reducing bacteria appear to be the best candidates for this process. Sulfate reducing bacteria constitute a group of anaerobic bacteria which used sulfur and its oxidized forms as electron acceptors. This sulfur 'respiration' is performed with very low redox potential cytochromes, cytochromes c_3, which, besides their role in electron transfer from an energetic substrate, are able to reduce various metals. Moreover, sulfate respiration produces sulfide which can easily reduce a large number of heavy metals and precipitate them as metallic sulfides [9]. The enzymatic reduction of Cr(VI) by *Desulfovibrio vulgaris* strain Hildenborough (DvH) has been recently studied [10, 11] and has demonstrated the involvement of two periplasmic electron transfer proteines, cyrochrome c_3 and hydrogenases, which are both universally found in sulfate reducing bacteria. The efficiency of this strain to reduce Cr(VI) allows to consider its use in waste water treatment for removing chromium and other heavy metals from solutions. In order to improve this process, recent studies were performed concerning the behaviour of DvH in a chromium contaminated medium [11].

In this field, isothermal microcalorimetry appears to be a powerful tool because it allows to monitor the effect of substances on the bioenergetic metabolism and growth of living cells. Indeed, this technique provides a continuous measurement of the heat production issued from a cellular suspension in a complex medium and thereby gives information in both qualitative and quantitative ways on its biological activity. Thus, heat production, as previously described, can be used for measuring the growth of microorganisms [12, 13]. Therefore, microcalorimetry has already been used for studying the effects of biocides or heavy metals on microbial activity [14, 15].

The present study was designed to evaluate by microcalorimetry the effect of Cr(VI) on DvH biological activity and, simultaneously, the reduction of this metal.

Experimental

Materials and methods

Cultivation procedure

DvH (DSM 644) was grown at pH 7.2 on a Starkey medium (SY) containing: NH_4Cl 37 mM, $MgSO_4 \cdot 7H_2O$ 8.1 mM, Na_2SO_4 28.1 mM, K_2HPO_4 2.8 mM, yeast extract (1 g L^{-1}), trace element solution (1 mL L^{-1}) [16] and sodium lactate 7.8 mM as carbon source and electron donor. After preparation SY was boiled, desaerated, cooled under Ar flow and conditioned under Ar atmosphere in 50 mL serum bottles in order to reach a –80 mV potential value. SY was sterilised by heat (110°C during 1 h). Cultivation was done at 37°C. Growth was monitored spectrophotometrically at 600 nm. Biomass was determined by total protein measurement using the Bradford assay with bovine serum albumin as standard [17].

Sample preparation

Towards the end of growth, an aliquot (0.2 mg of total protein) was taken and centrifugated at 16 100×g during 5 min. Bacteria were suspended in 50 mL SY pre-heated at 37°C and containing 0 or 75 μM Cr(VI). This suspension constituted the sample for the microcalorimetry experiment.

Microcalorimetry

Microcalorimetry experiments were conducted as follow in a heat-flow micro-calorimeter (Thermometric 2277 Thermal Activity Monitor) thermostated at 37°C: a 4 mL stainless steel ampoule was cleaned by sonication (5 min in Transsonic 080), washed with Neutracon 1%, dried and finally sterilized with a flame. Under sterile conditions and a N_2 flow, 3.8 mL of the sample, prepared as described above, were transfered into the ampoule which was then sealed and lowered in two steps into the measuring position of the calorimeter. A similar ampoule containing the same volume of sterile SY serves as reference. Data corresponding to the bacterial heat production were collected and analysed with Digitam v4.1 software.

Cr(VI) reduction analysis

Cr(VI) reduction was monitored by using a classical colorimetric method previously reported [18]. Briefly, 100 μL of suspension was added to 500 μL 1,5-diphenyl carbazide (10^{-6} M) solubilized in acetone. Next, the solution was acidified with 900 μL H_2SO_4 0.1 M and gently mixed. After 5 min, the absorption was measured at 540 nm. The Cr(VI) concentration was determined by refering to a calibration curve established under the same experimental conditions by using a standard Cr(VI) solution.

Results and discussion

Figure 1 shows the curves associated with the growth of DvH on SY in abscence of Cr(VI). In our experimental conditions, where lactate is used as the energy source at a concentration (7.8 mM) which is growth limiting, the shape of the power-time curve observed is specific of this strain. This curve can be divided into three phases that are lag phase, log phase and decline phase. We can observe that DvH develops a peak power value of 60 μW with a fast return to the baseline. As in the log phase of growth the cell number and culture time correspond to an exponential law [12], if the cell number is n_0 at time 0 and n_t at time t, then:

$$n_t=n_0e^{kt}$$

where $k(h^{-1})$ is the growth constant rate.

By considering that heat flux of each cell is w, we can write:

$$n_tw=n_0we^{kt}$$

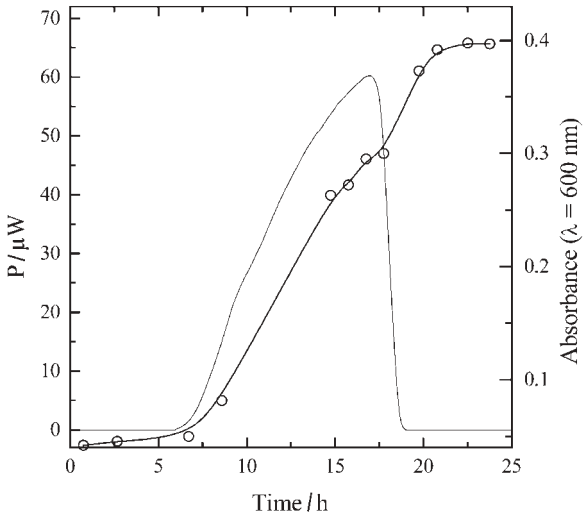

Fig. 1 Power-time and growth curves for cells of DvH growing anaerobically in SY medium with lactate limitation (7.8 mM). —— heat output (P); –o– absorbance (λ=600 nm)

In this condition, the power output is $P_0=n_0w$ and $P_t=n_tw$ and, thus, the thermogenesis curve of the log phase of growth can be represented according to the following equation:

$$P_t=P_0e^{kt}$$

And corresponding heat Q_t (J) is expressed by:

$$Q_t=(P_0/k)e^{kt} \quad (1)$$

As shown in Fig. 2, the integral representative curve is closely associated with the absorbance curve corresponding to the growth of DvH. Therefore the integral curve can be fitted with Eq. (1). The growth rate constant k, thus obtained, allows the calculation of the generation time ($t_{1/2}$), which is equal to (ln2)/k. In addition, the integral value Q=1.7±0.1 J permits the determination of the enthalpy associated with the metabolism of lactate (ΔH_{met}). The experimental values, thus obtained, are reported in Table 1.

The generation time mean value (3.4±0.4 h) was found slightly higher than the one previously determined (3.1±0.5 h) [19]. This can be due to experiments which were not performed under the same temperature conditions. Indeed, in our case, the growth was conducted at 37°C, whereas the previous one was performed at 30°C.

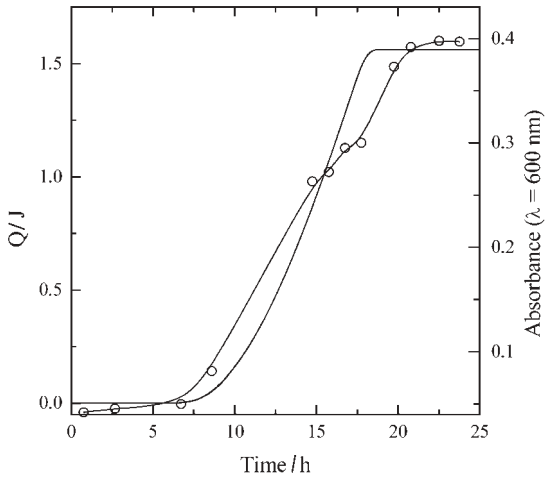

Fig. 2 Integral representative curve and growth curve obtained with DvH grown on lactate (7.8 mM). —— integral curve (Q); –o– absorbance (λ=600 nm)

Table 1 Experimental and calculated enthalpies associated with metabolism of lactate (ΔH_{met}) and generation time values ($t_{1/2}$)

	ΔH_{met}/kJ mol^{-1}	$t_{1/2}$/h
Experimental[a]	–56.3±5.7	3.4±0.4
Calculated	–66.8	3.1±0.5[b]

[a]Values are the means of four experiments
[b]Value is from [19]

For enthalpy associated with metabolism of lactate, the experimental mean value of ΔH_{met} (–56.3±5.7 kJ mol^{-1}) is in agreement with the classical stoichiometry reaction proposed for lactate oxidation [20]:

$$CH_3CHOHCOO^- + 0.5SO_4^{2-} + 0.71H^+ \rightarrow CH_3COO^- + CO_2 + 0.282HS^- + 0.218H_2S + H_2O$$

Indeed, considering the enthalpy of formation values listed in Table 2 and the heat of phosphate buffer ionization of 2.98 kJ mol^{-1} [19], the enthalpy associated to the catabolism of lactate (ΔH_{cat}) can be found equal to –70.3 kJ mol^{-1}, according to following equation.

$$\Delta H_{cat} = (\Delta H^{\circ}_{f,CH_3COO^-} + \Delta H^{\circ}_{f,CO_2} + 0.282\Delta H^{\circ}_{f,HS^-} + 0.218\Delta H^{\circ}_{f,H_2S} + \Delta H^{\circ}_{f,H_2O}) -$$
$$(\Delta H^{\circ}_{f,CH_3CHOHCOO^-} + 0.5\Delta H^{\circ}_{f,SO_4^{2-}} + 0.71\Delta H^{\circ}_{\text{ionization buffer}})$$

Since it has been reported [19] that ΔH_{met} can be related to this catabolic reaction by the following equation:

$$\Delta H_{met} = (1-\alpha)\Delta H_{cat} + Y_s \Delta h_{an}$$

where α represents the fraction of the energy source which is incorporated into the cellular material, Y_s is the molecular growth yield, and Δh_{an} is the enthalpy variation linked to the synthesis of 1 g (dry mass) bacteria, ΔH_{met} is calculated by considering the previously determined values for α (0.045 mol), Y_s (6.7 g mol^{-1}) and Δh_{an} (0.05 kJ g^{-1}) [19]. ΔH_{met} was found equal to –66.8 kJ mol^{-1} (Table 1).

Table 2 Values of the standard enthalpies

Compound	$\Delta H^o f$/25°C, kJ mol^{-1} [a]
$CH_3CHOHCOO^-$	–685.9
CH_3COO^-	–413.4
CO_2	–484.8
H_2O	–285.5
H_2S	–39.9
HS^-	–28.4
SO_4^{2-}	–892.6

[a]Values are from [19]

Figure 3 shows the power-time curve associated with the reduction of Cr(VI) (75 μM). By comparison with the curve obtained without Cr(VI) some marked differences can be observed. The power-time curve presents four phases. The length of the lag phase suggests that the retarding time of DvH is longer with the presence of Cr(VI). The log phase is seriously altered and no variation of absorbance is observed (data not shown). These indicate an inhibition of growth. A new phase appears; it corresponds to a slower increase of the heat output to yield a lower peak power value of

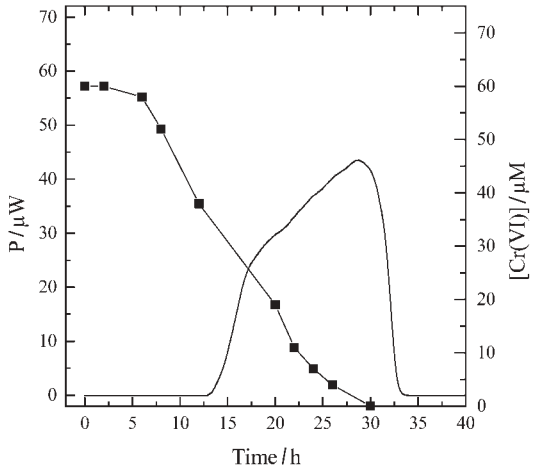

Fig. 3 Power-time curve for DvH in SY medium (lactate 7.8 mM) loaded with Cr(VI) 75 μM and kinetic reduction of Cr(VI) to Cr(III). —— heat output (*P*); –■– Cr(VI) concentration

44 μW. The decline phase is also altered, with a slower return to the baseline. These sound modifications of the whole curve indicate that Cr(VI) and/or Cr(III) induce an effect on the DvH metabolism. Consequently, the equations mentionned above are not applicable in this case. Determination of the generation time is thus precluded, and the integral value Q =2.1±0.1 J cannot be thermodynamically related to the classical metabolism of lactate as described above.

Even if chromium seems to exert a toxic but non letal effect on DvH, the bacteria are able to reduce Cr(VI). Indeed, results in Fig. 3 show that reduction starts about 6 h after inoculation and ends at about 30 h. The Cr(VI) reduction is observed during the lag phase of the curve. This indicates that the initial amount of bacteria (4 mg mL^{-1} total protein) is sufficient to initiate the reduction process. In addition, this one does not seem associated with growth. However it is possible that modifications of the metabolism may be implicated in the reduction of Cr(VI) to Cr(III).

To conclude, microcalorimetry allowed us to highlight the effects of chromium on DvH. Our results evidenced changes of the bacterial metabolism, alterations of the growth and indicate that most of the reduction activity occurred during the lag phase. In order to specify the molecular mechanisms implicated in the reduction power of DvH, studies are in progress in order to identify the metabolic pathways induced by the metal reduction and to investigate whether Cr(VI) serves as terminal electron acceptor during anaerobic respiration. However, there is no evidence showing that Cr(VI) yields energy to support anaerobic growth.

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