Biochimica et Biophysica Acta 816 (1985) 77–82
Elsevier

BBA 72630

Biotin uptake: influx, efflux and countertransport in *Escherichia coli* K12

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(Received February 1st, 1985)

Key words: Biotin transport; Affinity labeling; Kinetics; (*E. coli*)

Biotin uptake by *Escherichia coli* K12 has been reinvestigated. The vitamin uptake is an active process depending on energy and inhibited by uncouplers. The kinetic parameters ($K_m = 0.27$ μM, $V_{max} = 6.8$ pmol/min per mg dry cells) are close to those previously determined for a biotin-dependent strain *E. coli* C162 (Piffeteau, A., Zamboni, M. and Gaudry, M. (1982) Biochim. Biophys. Acta 688, 29–36). By use of biotin *p*-nitrophenyl ester, an affinity label of the biotin transport system, it was shown, under conditions of steady state, that the efflux of biotin is not energy dependent and is mainly mediated by a diffusion mechanism. Reexamination of the regulation of the biotin transport by biotin, revealed that only 50% of the biotin uptake system is under control by the vitamin.

Introduction

Biotin transport by microorganisms is now well documented and is in most cases an active process, i.e. an accumulation of the vitamin against a concentration gradient, energy dependent and carrier mediated [1]. However, divergent conclusions were reached about the biotin transport by *Escherichia coli*. Prakash and Eisenberg [2] concluded to an active process whereas Lichstein and Cicmanec [3] concluded to an energy independent process. More recently, Piffeteau et al. [4] reinvestigated this question with biotin-dependent *E. coli* strains and concluded to an active process. We have now extended this study to a *E. coli* K12 prototrophic strain with special emphasis on the mechanism and energy dependence of the biotin uptake, in particular on the efflux and countertransport of the vitamin by use of the *para*-nitrophenyl ester of biotin, a specific and covalently blocking agent for biotin transport [5,6].

Experimental

Materials

(+)-Biotin was a generous gift from Hoffmann La Roche Co. (Basel). [*carbonyl*-^{14}C]Biotin (50 Ci/mol) was purchased from Amersham International (U.K.) and [^{3}H]biotin (20 Ci/mmol) from C.E.A. (Saclay, France). (±)-Oxybiotin was a gift from Dr. Detitta (Buffalo) and (+)-α-dehydrobiotin was synthesized according to Field et al. [7,8]. Biotinyl *p*-nitrophenyl ester and [^{3}H]biotinyl *p*-nitrophenyl ester were prepared according to Wilchek and co-workers [5,9]. Mercaptoethanol and glucose were from Prolabo (Paris) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was purchased from Calbiochem.

E. coli K12 (54 117 Pasteur collection) was grown firstly on a complex medium (10 g Bactotryptone, 5 g NaCl, 5 g yeast extract per litre (pH 7.3)) then on a synthetic medium 63 (13.6 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot 7\ H_2O$,

0.5 mg $FeSO_4 \cdot 7H_2O$ per litre; adjusted to pH 7.0 with 4 M potassium hydroxide) [10] supplemented with 2 g glucose and 35 mg L-histidine per litre. When necessary, biotin was added aseptically after autoclaving. Cell growth was monitored at 570 nm ($A_{570} = 1$ corresponding to $7.5 \cdot 10^8$ cells per ml).

Methods

Uptake studies. Cells were harvested (10000 × *g*, 15 min, room temperature) in the mid exponential phase ($5.5 \cdot 10^8$–$7 \cdot 10^8$ cells/ml), washed with medium 63 (room temperature) and suspended in uptake medium (13.6 g KH_2PO_4, 2.6 g K_2SO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.5 mg $FeSO_4 \cdot 7H_2O$, 3.6 g glucose, 0.1 g chloramphenicol per litre; adjusted to pH 6.6 with 4 M potassium hydroxide; room temperature) at a concentration of $3.7 \cdot 10^9$ cells/ml. After equilibration at 37°C for 10 min, the uptake experiments were initiated by addition of labelled biotin (0.328 μM). As previously described [4], 1 ml samples were pipetted, rapidly filtered and washed and the radioactivity incorporated by the cells estimated in an SL 30 Intertechnique scintillation counter in Bray's liquor [11]. Correction for quenching was achieved by the double channel method.

Incubations with biotinyl p-nitrophenyl ester. Bacteria ($3.7 \cdot 10^9$ cells/ml) were incubated in a glucose free uptake medium at 37°C for 30 min in the presence of biotinyl *p*-nitrophenyl ester (0–23.6 μM). They were harvested and washed twice with uptake medium and assayed for biotin uptake (vide supra). After incubation with [^{3}H]biotinyl *p*-nitrophenyl ester (1 Ci/mmol, 28 μM) the incorporation was determined as a function of time by filtration of bacteria as in biotin uptake experiments.

Influence of biotinyl p-nitrophenyl ester on the efflux of biotin. Bacteria ($3.7 \cdot 10^9$ cells/ml) were incubated at 37°C in the uptake medium in the presence of [^{3}H]biotin (0.328 μM). After 30 min, biotinyl *p*-nitrophenyl ester was added (6.6 μM) and the incubation continued for 30 min. An excess of biotin (6.15 μM) was added and the efflux of radioactivity monitored as above by filtering cells.

Influence of growth medium biotin concentration on biotin uptake. Synthetic growth medium supplemented with increasing amounts of biotin (0–123 nM) was inoculated with an overnight culture of bacteria in low biotin concentration medium (0.41 nM) to a concentration of 10^8 cells/ml. Bacterial growth was monitored and the bacteria were harvested in the mid log phase ($6 \cdot 10^8$ cells/ml) and were tested for biotin uptake as above.

Results and Discussion

Features of E. coli K12 biotin uptake

Biotin uptake was studied with bacteria grown in a biotin free synthetic medium 63 [10] supplemented with glucose. The cells were harvested in the middle of the exponential phase since we observed previously [4] that the growth phase has a large influence on the biotin uptake. Incubation of resting cells was routinely carried out at 37°C with 0.328 μM biotin. Fig. 1 illustrates the features of *E. coli* K12 biotin uptake. The uptake was linear for less than 2 min and reached a maximum between 15 and 20 min. This maximum corresponded to 10 pmol of biotin per mg of dry bacteria, i.e. about 33% of the total biotin content of auxotrophic *E. coli* C162 [4]. In contrast with

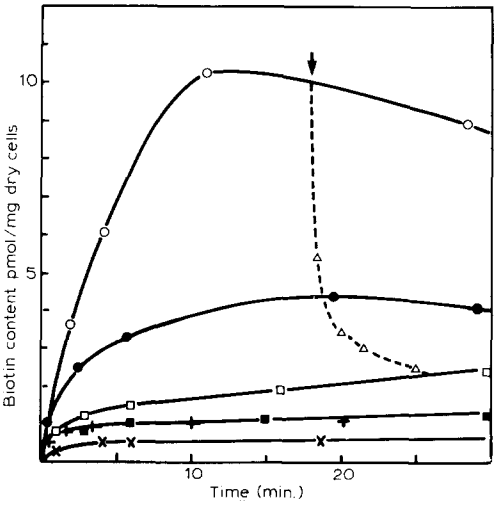

Fig. 1. Uptake of biotin by *E. coli* K12 resting cells. *E. coli* K12 cells grown on synthetic medium supplemented with glucose were resuspended in the uptake medium ($3.7 \cdot 10^9$ cells/ml). Incubations were carried out at 37°C (○, glucose 3.6 g/l; ●, no glucose; +, glucose 3.6 g/l CCCP 10 μM; ×, no glucose CCCP 10 μM) or at 4°C (□, glucose 3.6 g/l; ■, no glucose). Uptake was initiated with [^{14}C]biotin (0.328 μM). After loading of bacteria at 37°C, addition CCCP (⬇) (10 μM) induced the efflux of biotin (△).

this auxotrophic strain, an overshoot phenomena was frequently observed but with a lower amplitude than with *Saccharomyces cerevisiae* [12]. The biotin uptake was temperature dependent and energy dependent. It was very low at 4°C (2 pmol/mg dry bacteria), depended on the presence of glucose and was inhibited by uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) or by energy inhibitor sodium azide. Addition of CCCP to a suspension of cells loaded with biotin resulted in a very rapid loss of internal biotin down to the level observed for an uptake at 4°C. The behaviour of the *E. coli* K12 biotin pool was reminiscent of that of the free biotin pool (i.e. not linked to carboxylases) of the auxotroph *E. coli* C162 which we studied previously [4]. This was confirmed by the counterflow experiments illustrated in Fig. 2. After loading with labeled vitamin (0.328 μM) the countertransport was initiated by adding an excess of unlabeled biotin (6.15 μM) (Fig. 2a) or, in a complementary experiment, after loading the cells with unlabeled biotin (0.328 μM) by adding labeled biotin (0.328 μM) (Fig. 2b). Fig. 2a illustrates the counterflow of biotin while Fig. 2b illustrates the flow of biotin inwards the cell at equilibrium in both experiments. This revealed that the concentration of biotin in the free pool corresponds to a dynamic equilibrium between the influx and the efflux of the vitamin. The similarity of the efflux induced by an excess of external biotin or by addition of uncouplers is consistent with a scheme where the influx is dependent on energy, whereas the efflux is not [4]. The kinetic parameters derived from initial rate measurements ($K_m = 270 \pm 60$ nM and $V_{max} = 6.8$ pmol/min per mg dry weight) are very close to those determined for *E. coli* Y10 ($K_m = 140$ nM, $V_{max} = 6$ pmol/min per mg dry weight) [2] and the inhibition by biotin analogues exhibit the same characteristics as those determined with the auxotroph *E. coli* C162 [4]: the α-dehydrobiotin was a competitive inhibitor (Fig. 3) although with a lower relative affinity (K_i(α-dehydrobiotin)/K_m (biotin) = 10) with the prototroph than with the auxotroph (K_i(α-dehydrobiotin)/K_m(biotin) = 5) [4], and oxybiotin was a very poor non competitive inhibitor.

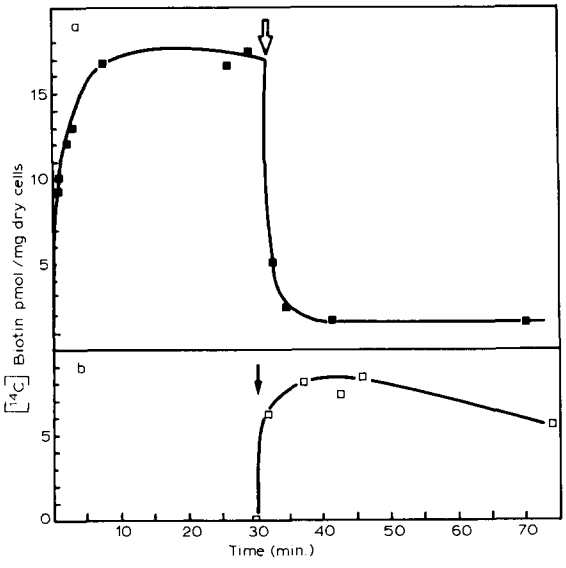

Fig. 2. Exchange of intracellular biotin by *E. coli* K12 resting cells. The same suspension of *E. coli* K12 cells ($3.7 \cdot 10^9$ cells/ml) grown on a synthetic medium was used in both experiments. (a) ■, after incubation with [^{14}C]biotin (0.328 μM), unlabeled biotin (6.15 μM) was added (⇓). (b) □, after incubation with biotin (0.328 μM), [^{14}C]biotin (0.328 μM) was added (⇓).

Study of the biotin transport system of E. coli K12 with biotinyl p-nitrophenyl ester

Biotinyl *p*-nitrophenyl ester has been used by Wilchek and co-workers to study the biotin trans-

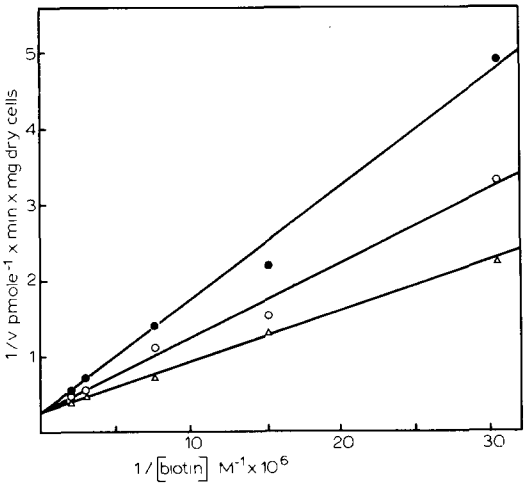

Fig. 3. Inhibition of biotin uptake by α-dehydrobiotin. The uptake of biotin and the inhibition by α-dehydrobiotin was studied with a suspension of *E. coli* K12 cells ($3.7 \cdot 10^9$ cells/ml) grown on synthetic medium. Two concentrations of α-dehydrobiotin were used (△, 0; ○, 1.65 μM; ●, 3.3 μM). Cells were incubated for 2 min before filtration and washing and counting.

port system of *S. cerevisiae* [5,6,9]. This derivative is an affinity label of the system and upon incubation, biotin becomes linked covalently to an essential transport protein. Furthermore, the covalent attachment of biotin to the carrier can be split by treating with thiols, restoring the ability of the yeast cells to transport biotin [9].

We observed that incubation of *E. coli* K12 cells with biotinyl *p*-nitrophenyl ester for 30 min resulted in an inhibition of biotin uptake: 53% and 86%, respectively, at biotinyl ester concentrations of 6.6 μM and 26.3 μM. These figures are in good agreement with those obtained with *S. cerevisiae* [5]. However, as shown in Fig. 4 we were unable to restore the biotin uptake after inhibition with the biotinyl-activated ester, by incubation with mercaptoethanol as observed with *S. cerevisiae* [9]. Furthermore, in agreement with former studies [13], we observed that mercaptoethanol inhibited

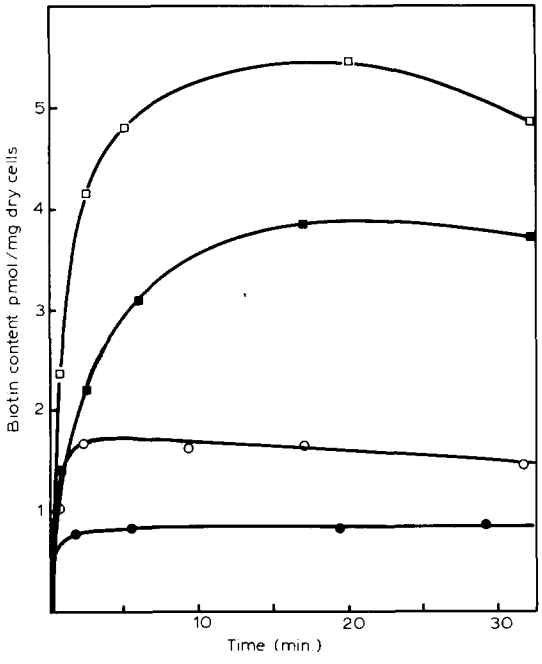

Fig. 4. Influence of biotinyl *p*-nitrophenyl ester and mercaptoethanol on biotin uptake by resting cells of *E. coli* K12. The same suspension of *E. coli* K12 cells ($3.7 \cdot 10^9$ cells/ml) grown on synthetic medium was used in all the experiments. [^{3}H]Biotin (0.328 μM) was added after two 30 min preincubation periods. □, no addition; ■, no addition, mercaptoethanol (0.15 M); ○, biotinyl *p*-nitrophenyl ester (28 μM), no addition; ●, biotinyl *p*-nitrophenyl ester (28 μM), mercaptoethanol (0.15 M).

significantly the biotin uptake (Fig. 4). As far as we used 'Viswanatha et al. regeneration conditions' [6] (i.e. treatment of biotinyl *p*-nitrophenyl ester inhibited cells with mercaptoethanol), the difference with Viswanatha et al. result that is the lack of biotin uptake system regeneration, originates certainly in the different types of microorganisms used.

In order to obtain more information on the labeling with biotinyl activated ester, we prepared it from [^{3}H]biotin. The labeling depended on the concentration of the active ester and ranged from 8.2 to 12 nmol/mg dry cells for respective ester concentrations of 7 to 27 μM. The labeling reached a plateau level (constant till 30 min) within 30 s and did not depend on energy: uncouplers or absence of glucose did not modify its value. Moreover unexpectedly, an excess of biotin did not protect significantly the cells against labeling. All these arguments suggested that although the biotinyl *p*-nitrophenyl ester inhibits the biotin uptake, i.e. labels specifically some constituent of the biotin transport system, most of the labeling of the intact *E. coli* K12 cell is not specific, in contrast with the specificity observed with *S. cerevisiae* by Wilchek and collaborators [5,6,9]. This labeling could not be used to visualize and study quantitatively the *E. coli* biotin uptake system.

However, we decided to take advantage of the specific part of the labeling, although it represented only a low percentage, to get more information concerning the biotin transport system, and more particularly to determine if biotin is using the same system for its influx and efflux.

Following loading the cells with radioactive biotin (0.328 μM) for 35 min, biotinyl *p*-nitrophenyl (28 μM) was added and the biotin content of the cells monitored. 30 min after the addition of the ester, biotin in excess was added (6.15 μM) in order to exchange the intracellular biotin (Fig. 5). The addition of the active ester induced a slow decrease of the biotin content originating very likely from the decrease of the number of transport systems that were irreversibly trapped by the affinity label. After addition of biotin in excess in the incubation and in the blank, the counterflow was monitored. Analysis of this part of the curves using a curve fitting program, yielded exponential decreases with constants of

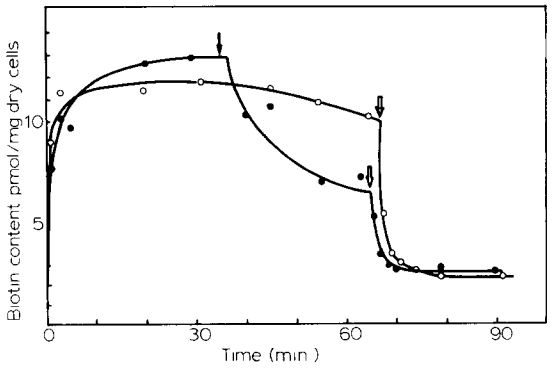

Fig. 5. Study of efflux of biotin with *E. coli* K12 resting cells using biotinyl *p*-nitrophenyl ester. The same suspension of *E. coli* K12 resting cells ($3.7 \cdot 10^9$ cells/ml) grown on synthetic medium was used in both experiments. [^{3}H]Biotin (0.328 μM) was added at $t = 0$. Biotinyl *p*-nitrophenyl ester (28 μM) was added after 35 min (⇓) in one incubation (●) and biotin in excess (6.15 μM) was added in both incubations (⇓) at 65 min (●) and 67 min (○).

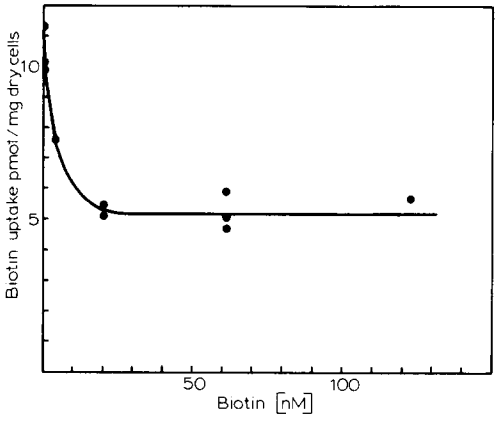

Fig. 6. Repression of biotin uptake. Growth media supplemented with biotin (0 → 123 nM) were inoculated ($7.5 \cdot 10^7$ bacteria/ml) with an overnight culture of *E. coli* K12 (synthetic medium, 0.4 nM biotin). The growth was monitored and cells were harvested when cultures reached $(5\text{–}6) \cdot 10^8$ bacteria/ml. Biotin uptake at the steady-state on resting cells was assayed as usual.

$5 \cdot 10^{-3}$ s^{-1} and $4.8 \cdot 10^{-3}$ s^{-1}, respectively, for the incubation with the active ester and for the blank.

The rate of efflux of biotin was thus unchanged whereas the number of elementary active uptake components was lowered. Moreover experiments with uncoupler suggested that the efflux is not energy dependent. It is thus probable that two systems, an energy-driven active uptake of biotin and a parallel diffusion are operating. Although both systems can intrinsically operate inward and outward, the concentration conditions ($[\text{biotin}]_{\text{inside}} \gg [\text{biotin}]_{\text{outside}}$) impose that the influx is mainly mediated by the active carrier and the efflux by diffusion.

Control of biotin transport: repression by biotin

Cicmanec and Lichstein noticed that biotin represses the biotin uptake system of *S. cerevisiae* [13] while comparing cells grown in high biotin (0.1 μM) or in low biotin (1 nM) media. The K_m was unchanged whereas the V_{max} was 35-times lower for the cells grown in the high biotin medium. Prakash and Eisenberg concluded also to repression in the case of *E. coli* [2] but in contrast with *S. cerevisiae* they observed that the uptake was only divided by two for a biotin concentration of 60 nM. We reinvestigated that question more thoroughly by growing derepressed cells (overnight culture in the presence of 0.4 nM biotin) in media provided with increasing amounts of biotin (0.4 nM → 123 nM). Fig. 6 illustrates the dependence of the plateau value (steady-state) for biotin uptake as a function of biotin concentration in the growth medium. The uptake K_m for cells grown in low (0.4 nM) or high biotin (123 nM) concentrations were identical (0.13 μM and 0.12 μM) whereas the V_{max} was approximatively divided by 2 (respectively, 5 and 1.94 pmol/min per mg dry cells). This confirmed that the uptake system is repressed by biotin, but only to about 50% of the derepressed level as observed by Prakash and Eisenberg [2]. Furthermore the maximum repression is reached for concentrations of biotin (20 nM) very similar to those which repress the synthesis of the biotin biosynthesis enzymes [14].

Acknowledgement

This work was supported by the Centre National de la Recherche Scientifique.

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