Carbon-13 labelled biotin – a new probe for the study of enzyme catalyzed carboxylation and decarboxylation reactions

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[2'-^{13}C]Biotin was incorporated into avidin (egg white), glutaconyl-CoA decarboxylase (EC 4.1.1.70) from *Acidaminococcus fermentans* and the biotin carrier of transcarboxylase from *Propionibacterium freudenreichii* (EC 2.1.3.1). ^{13}C-NMR measurements showed an upfield shift of the carbonyl carbon of 3.1 and 2.0 ppm for both enzymes, whereas binding to avidin induced no significant change of the chemical shift as compared to free biotin. The data indicate that the enzymes provide an electronic environment for the covalently bound biotin which favours carboxylation. In addition it was demonstrated by NMR-measurements that glutaconyl-CoA decarboxylase, from which the hydrophobic carboxy-lyase subunit (β) was removed, could carboxylate free biotin.

[2'-^{13}C]Biotin; Avidin; Glutaconyl-CoA decarboxylase; Transcarboxylase; NMR

1. INTRODUCTION

Biotin covalently linked to the ϵ-amino group of a lysine residue is the prosthetic group of a variety of enzymes, as are several ATP-dependent carboxylases, carboxyl transferases [1] and 3 sodium ion pumping decarboxylases [2]. It was established in some cases that during catalysis 1'-*N*-carboxybiotin is formed [1]. Despite this fact very little is known about the mechanism of carboxylation and CO_2-transfer within the active sites of these enzymes. *O*-Phosphorylated forms of biotin [3,4] as intermediates or attack of a biotin enol at carboxyphosphate have been discussed [5]. A chemically induced carboxylation yielding carboxybiotin in vitro has not been reported. In order to gain more direct insight into the nature of these processes, we have prepared [2'-^{13}C]biotin (Fig. 1) and report here our nuclear magnetic resonance measurements (NMR) of this prosthetic group in different protein environments.

2. MATERIALS AND METHODS

2.1. *Avidin biotin complex*

Avidin (20 mg, Boehringer Mannheim) was incubated with 2 mg [2'-^{13}C]biotin at pH 7.5, dialysed and concentrated to 0.4 ml by lyophilisation.

2.2. *Bacteria*

Acidaminococcus fermentans ATCC 25085 was grown on the glutamate medium [2] in which unlabelled biotin was replaced by 500 nM [2'-^{13}C]biotin. *Propionibacterium freudenreichii* subsp. *shermanii* strain 33 was a gift from Professor J. Rétey (Karlsruhe, Germany). It was grown anaerobically on the following medium at 30°C: 80 mM glucose, 0.5% yeast extract, 0.5% tryptone, 30 μM $Co(NO_3)_2$, 2 μM thiamin, 1 μM [2'-^{13}C]biotin and 30 mM K^+-phosphate, pH 7.7.

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2.3. *Preparation of [biotin-^{13}C]glutaconyl-CoA decarboxylase*

The enzyme was prepared as described in [6] whereby Triton was replaced by 0.05% Brij 35 in the elution buffer for the affinity column. The sample used for NMR-measurements contained 13 mg glutaconyl-CoA decarboxylase (90 U) in 0.5 ml. Another sample was treated with 4% 1-butanol for 1 h at 37°C in order to remove the β-subunit [7]. After centrifugation and dialysis it was concentrated to 0.5 ml (10 mg protein).

2.4. *Formation of 1'-N-carboxy[2'-^{13}C]biotin*

The incubation contained in a total volume of 900 μl at ambient temperature: 7 mg butanol treated glutaconyl-CoA decarboxylase (see above), 1 mM dithiothreitol, 1 mM EDTA, 0.11 mM acetylphosphate, 11 mM NAD, 13 mM CoASH, 13 mM glutaconate and 66 mM [2'-^{13}C]biotin. The reaction was started by addition of 0.3 mg (30 μl) auxiliary enzymes [2].

2.5. *Isolation of transcarboxylase*

The intact 25 S enzyme complex was isolated essentially as described in [8]. The complex was dissociated at high pH according to [9], and the 1.3 S biotin carrier subunit purified by anion exchange chromatography on Q-Sepharose FF (Pharmacia). The material was adsorbed on the column in 10 mM NH_4HCO_3, pH 9.0, and eluted at the same pH by a concave salt gradient from 10 to 500 mM NH_4HCO_3.

2.6. *Preparation of [2'-^{13}C]biotin and 1'-N-carboxybiotin*

[2'-^{13}C]biotin was prepared according to [10]; unlabelled carboxybiotin was prepared by hydrolysis of its dimethylester according to the procedures described in [11].

2.7 *NMR Measurements*

The NMR experiments were performed on a Bruker-AM 400 spectrometer in 5 mm sample tubes using phosphate buffered solutions with addition of 10% D_2O. Chemical shifts were measured vs internal

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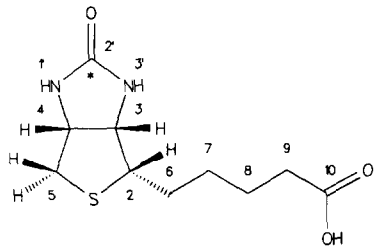

Fig. 1. Formula of [2′-^{13}C]biotin

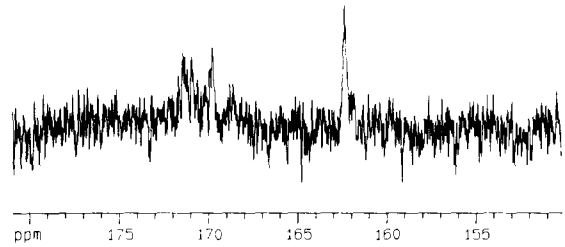

Fig. 2. ^{13}C-NMR spectrum of [2′-^{13}C]biotin (162.3 ppm), incorporated in glutaconyl-CoA decarboxylase (26 mg/ml, pH 6.8), signals at 171 ppm stem from the carbonyl carbon atoms of the enzyme.

dioxane and are reported vs tetramethylsilane by applying $\delta_{dioxane}$ = 66.6 ppm [12]. Typically the spectra were taken with a 45° excitation pulse width of 5 μs, composite pulse decoupling and an interpulse delay of 1.8 s on a time domain of 64k data points. Usually overnight runs of at least 12–16 h were necessary to provide sufficient signal to noise ratio. Experimental linewidths are reported after applying an exponential function with a line broadening factor of 3 Hz.

3. RESULTS AND DISCUSSION

Before incorporating biotin with a covalent bond into enzymes we first established the experimental feasability of our approach. It had to be demonstrated, that biotin bound to a protein gives a ^{13}C-NMR signal with a sufficiently small linewidth after a reasonable amount of measuring time. For the sake of simplicity we have chosen to examine the complex with avidin. The crystal structure of the close analogue streptavidin was recently communicated [13]. Much to our surprise the biotin-avidin complex yields a ^{13}C-NMR signal only 0.17 ppm upfield from the signal of free biotin (165.43 ppm) at pH 6.8 in D_2O solution, with a linewidth of 12 Hz. To prove that this signal originates from bound [2′-^{13}C]biotin we added free [2′-^{13}C]biotin in excess which resulted in two signals. Whether the close interaction indicated from the crystal structure of streptavidin between tyrosine 33 and biotin does not affect the chemical shift, or other amino acids in the vicinity of the amide nitrogen atoms of biotin counterbalance a complexation shift, cannot be distinguished at present. The linewidth is in accordance with the expectation from an estimation of the rotational correlation time [14] using a molecular mass of 68 kDa.

Whereas the biotin-avidin complex does not show a significant chemical shift change compared with free biotin, we found that the signal of [2′-^{13}C]biotin covalently bound to glutaconyl-CoA decarboxylase is shielded by 3.1 ppm and resonates at 162.3 ppm. The linewidth of the signal bound to this enzyme (molecular mass ca 100 kDa) is 10 Hz and thus smaller than the linewidth of biotin in the avidin complex (68 kDa), indicating perhaps an extra amount of motional freedom of the head group. A typical spectrum of the glutaconyl-CoA decarboxylase with incorporated [2′-^{13}C]biotin is shown in Fig. 2.

However, the significant shielding of the signal requires a specific interaction with the protein. To assure that this signal stems from bound [2′-^{13}C]biotin we have subsequently run digestion experiments with trypsin followed by acidification with HCl (pH 1) within the NMR tube, and shown that finally the signal returns to its original position. Removal of the sodium ion pumping subunit (β) [6,7] alone does not affect the chemical shift .

Excess biotin can be carboxylated by glutaconyl-CoA decarboxylase without being covalently bound to the enzyme [6]. To confirm these findings by NMR, we measured free [2′-^{13}C]biotin in the presence of glutaconyl-CoA decarboxylase, the β-subunit of which was previously removed, acetyl-CoA, glutaconate CoA-transferase and glutaconic acid as CO_2 source. Indeed, after about one hour of measurement, we could detect carboxybiotin. The ^{13}C chemical shift of carbon atom 2′ at 160.4 ppp was confirmed by preparing carboxybiotin via organic synthesis. We note that the correct structure of this compound (it is the question whether N-1 or N-3 is carboxylated) was ascertained by detecting a $^3J_{H,H}$ spin, spin coupling between H-3 and H-3′ via a long range COSY experiment. To our knowledge this is the first time, where the enzymatic carboxylation of biotin was directly demonstrated by NMR.

The linewidth of the [2′-^{13}C]biotin in the biotin carrier subunit of transcarboxylase (12 kDa) was almost as small as that of free biotin (5 Hz) indicating a high mobility of the prosthetic group in this protein. However, as in glutaconyl-CoA decarboxylase, we detected a 2.0 ppm upfield shift (163.4 ppm). This indicates that the environment of the biotin urea head group is similar in both proteins. This upfield shift indicates that the urea group of biotin has to experience an electronic change to become functional in carboxylation or decarboxylation reactions. Carboxylation experiments with [2′-^{13}C]biotin bound to transcarboxylase are in progress.

In summary our results show, that [2′-^{13}C]biotin or its ^{15}N labelled isomer [15] are promising new probes to study binding interactions with proteins and offer the possibility to study enzyme catalyzed reactions.

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