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Determination of the binding specificity of the 12S subunit of the transcarboxylase by saturation transfer difference NMR

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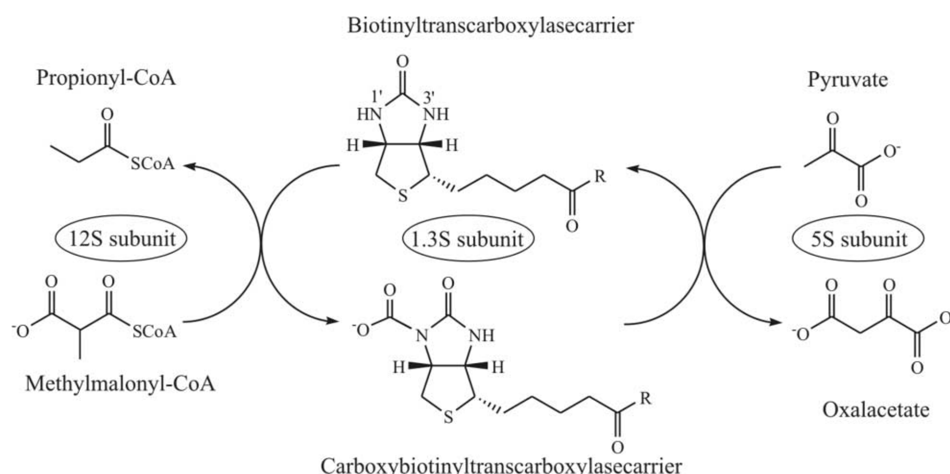
In this study we present the characterization of the interaction of biotin and methylmalonyl-CoA (MMCoA) with the carboxyltransferase subunit (12S) from the transcarboxylase (TC) from *Propionibacterium shermanii*. This biotin dependent multienzyme complex catalyses the transfer of carbon dioxide from methylmalonyl-CoA (MMCoA) to pyruvate. The Saturation Transfer Difference NMR (STD) technique was performed to determine the binding epitope from biotin and MMCoA to the 12S subunit. We could show by titrations during STD experiments that biotin and MMCoA bind cooperatively in one binding pocket.

Introduction

Biotin dependent carboxylases play an important role in the mammalian metabolism. For example, acetyl-CoA carboxylase (ACC) catalyses the essential step in the biosynthesis of fatty acids.[1] There are three different types of biotin dependent enzymes. Class I enzymes are the carboxylases, which require ATP and Mg^{2+} for the transfer of CO_2 from hydrogen carbonate to metabolites. Class II enzymes couple two carboxylation reactions and the transcarboxylase (TC) (EC 2.1.3.1) from *Propionibacterium shermanii* is the only known enzyme of this class. The sodium ion transport decarboxylases form class III. These enzymes utilize the free energy of decarboxylation to build up a Na^+ gradient. The common mechanism of biotin dependent enzymes is that the reaction takes place in two separate subsites in a two-step reaction. This has been known for a long time, but the detailed mechanism as to how biotin is converted into carboxybiotin or how biotin is involved in the enzymatic environment is still under discussion.[2–5]

TC is a 1200 kDa multi enzyme complex, composed of 30 separable polypeptides, which forms three different structural and functional subunits. The core of the enzyme is the cylindrical hexameric 12S subunit (60 kDa per 12S monomer). The bottom and the top of the 12S subunit are surrounded by six 5S subunits, being homodimers (116 kDa).[2,6] Each 5S and 12S subunit are connected *via* a linker, the 1.3S subunit forming a 123 amino acid monomer (12 kDa).[7] This transcarboxylase carrier (tcc) is known to transport carbon dioxide from methylmalonyl-CoA (MMCoA) at the 12S subunit to pyruvate at the 5S subunit (Scheme 1). On this carrier the biotin is covalently attached to the ε-amino group of Lys-89, which lies within the conserved amino acid sequence Ala-Met-Lys-Met.[7,8] The structure of the tcc, solved by NMR spectroscopy, shows a high internal 2-fold symmetry.[9,10] The C-terminal domain folds in a compact four-stranded antiparallel *β*-sheet. The same folding pattern is found in the structure of the biotin carboxycarrier subunit (BCCP) from ACC from *E. coli*.[11–13] The structure comparison reveals for the BCCP a thumb/loop motif, which is missing in the tcc. This thumb is responsible for the protein–biotin interaction in the BCCP. In contrast, there is no detectable interaction between the 1.3S subunit of TC and biotin, but the biotin is presented by its tcc essentially free to the environment. This led to the question, whether the 12S subunit could also carboxylate free biotin without the help of the tcc, although earlier work showed that the whole TC complex was not able to perform this reaction.[14] If one finds a binding pocket for biotin then one can go further to study carboxylation of free biotin which is not more bonded to a subunit. The idea is based on the work of Lynen, who was able to show carboxylation of free biotin with β-methyl-crotonyl-CoA carboxylase.[15] The structure of the carboxyltransferase subunit (12S) from TC has been solved by X-ray crystallography.[16] It only shows the MMCoA binding pocket but does not give any information about a biotin binding motif on this subunit.

The present study reports the binding specificity and the group epitope mapping of biotin and MMCoA to the 12S



Scheme 1 Carboxyl transfer catalysed by transcarboxylase from *Propionibacterium shermanii*.

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subunit of the TC obtained by Saturation Transfer Difference NMR (STD).[17–19] With this study we hope to get a deeper insight into the carboxylation mechanism of TC.

Experimental

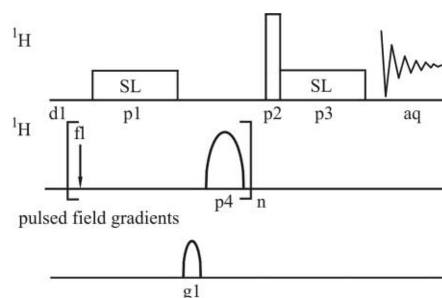
Sample preparation

Biotin and MMCoA were purchased from Sigma. The 12S subunit was obtained *via* the expression and purification protocol according to Woo *et al*. and Wang *et al*. but with some modification.[20,21] *Escherichia coli* JM109, transformed with the construct encoding the 524 residue of the 12S polypeptide, was grown overnight at 37 °C with a constant shaking of 250 rpm in 2×YT-Medium containing ampicillin (100 µg ml^{-1}). The culture was then diluted 1 : 100 to a total volume of 6 L. After about 4 h of growing, when the OD_{600} reached about 0.8, the expression was induced with 1 mM IPTG (isopropyl-*β*-D-thio-galactopyranoside). After about 6 h of growing the cells were harvested by centrifugation (5000 g for 15 min). The pellet was washed twice in 30 ml 25 mM potassium phosphate buffer at pH 7.2 containing 0.1 mM NaN_3, 1 mM DTT (1,4-dithio-DL-threitole), 0.1 mM EDTA (ethylenediaminetetraacetic acid) and 0.1 mM PMSF (phenylmethanesulfonyl fluoride) freshly added and then resuspended in the same buffer. All the purification steps were carried out at 4 °C and all the buffers used contained the additives mentioned above. The cells were lysed three times *via* french press. Cellular debris were removed by centrifugation (25000 g for 30 min). After a 45% ammonium sulphate precipitation the supernatant was dialyzed for 12 h against 5 L (2 × 2.5 L) of 25 mM potassium phosphate buffer at pH 7.2. The purification of the protein was done by anionic exchange, cationic exchange and size exclusion chromatography. The dialysate was applied to DEAE-cellulose (Sigma) column (30 × 5 cm) and eluted with a three step gradient of potassium phosphate buffer at pH 7.2 of 100, 200 and 500 mM. The fractions containing the 12S protein (500 mM eluate) were identified by SDS polyacrylamide gel electrophoresis and pooled and subjected to 45% ammonium sulphate precipitation. The supernatant was dialyzed for 12 h against 5 L of 25 mM potassium phosphate buffer at pH 6.5 and applied to cellulose phosphate (Sigma) column (20 × 5 cm). The protein was eluted in a four step gradient of potassium phosphate buffer at pH 6.5 of 100, 200, 500 and 750 mM. The major protein fractions (500 mM eluate) were pooled and concentrated by centrifugation with a Centricon (Millipore) membrane having a cut-off value of 50 kDa. The protein was passed over a Superdex, 200 prep. grade (Amersham Biosciences) column (70 × 2.5 cm). The elution was carried out with 250 mM potassium phosphate buffer at pH 6.5. The 12S subunit was eluted as the second peak and was concentrated like mentioned before. The typical recovery was 6–7 mg of 12S from 13–14 g of cell paste. The protein concentration was determined by a Bradford assay.

NMR samples were prepared by buffer exchange into 600 µl of 99.9% D_2O containing 0.5 M phosphate buffer at pH* 6.6 (not corrected for D_2O) with a protein concentration ranging between 0.05 and 0.08 mM.[17]

NMR experiments

All NMR experiments were performed on a Bruker Avance 700 MHz spectrometer equipped with a 5 mm inverse triple-resonance probe head at 278 K or at 298 K system temperature. The pulse scheme of the 1D STD NMR spectra corresponds to Mayer and Meyer,[17] but for the epitope mapping, a pulse scheme with slight modification for a better phase behaviour, with respect to the difference formation, was applied (Scheme 2).[22] The on-resonance irradiation of the protein was performed at a chemical shift of 0.4 ppm (for biotin) or −0.4 ppm (for MMCoA). Off-resonance irradiation was applied at 30 ppm, where no protein signals were present. The 1D STD spectrum was generated internally *via* phase cycling after every scan. The protein was selectively irradiated by a train of 40 Gauss–shaped 90° pulses of 50 ms length each with 1 ms intervals leading to a total saturation time of 2.04 s. The first spin-lock pulse and the gradient were used for suppression of unwanted signals, the $T_{1\rho}$ filter with a 30 ms spin-lock pulse was applied to suppress the protein resonances. All STD spectra were recorded with 3 k or 12 k transients. The spectra were referenced using the water signal set to 4.8 ppm. The ^1H spin-lattice relaxation times were measured using the inversion recovery sequence.



Scheme 2 Pulse sequence for the 1D STD NMR experiments. The reference spectrum is achieved *via* phase cycling after every scan. The irradiation frequency of the on-resonance pulses is applied at 0.4 ppm for biotin and −0.4 ppm for MMCoA. The off-resonance irradiation is performed at 30 ppm. The selective irradiation is achieved by a train of 40 Gauss–shaped 90° pulses of 50 ms length each with 1 ms intervals corresponds to $\gamma B_2 \approx 120$ Hz. The spinlock pulses p1 and p3 have an intensity $\gamma B_1 \approx 8000$ Hz. The gradient strength is 0.14 T m^{-1}. The phases are: p1 = (x); p2 = 2(x), 2($-x$), 2(y), 2($-y$); p3 = 4(y), 4($-x$), 4($-y$), 4(x); p4 = (x); acquisition (aq) = (x, $-x$, $-x$, x, y, $-y$, $-y$, y).

Results and discussion

Binding epitope mapping of biotin to the 12S subunit of the TC

First we focused our studies on the protein ligand interaction between the 12S subunit and biotin to test whether there is a biotin binding pocket on this unit or not. A high ligand excess was used for a better STD effect and hence for a better recognition of the ligand protons near or far away from the protein.[18,19] Fig. 1 displays the reference NMR spectrum (A) of biotin and the 12S subunit in a ratio of 1 : 100, the corresponding STD NMR spectrum (C) and the control STD NMR spectrum (B) of 5 mM biotin without protein at 278K. This observation indicates that there has to be a binding contact, because all the biotin resonances appear in the STD NMR spectrum. The protons of the ligand, which are closer to the protein experience a larger saturation transfer, hence they give a higher integration value. The H4 proton of biotin, which gives the largest integral value, was set to 100%. In Fig. 1D the relative quantity of saturation is shown for the individual protons. The bridgehead atoms H4 and H3 give the highest effect. The diastereotopic protons 5a and 5b differ in the STD intensities by about 30 percentage points, where the proton 5b, situated on the opposite side of the bridgehead atoms, shows with 72%, a higher value than the proton 5a with 44%, being situated on the same side. The H2, H7, H9 and H6/H8 valeryl side chain protons have comparable STD effects ranging between 55% and 66%. Due to overlapping of the signals H6 and H8, they were treated as one group to calculate the relative STD intensity. The obtained binding epitope map must be interpreted with caution. The T_1 relaxation times of biotin (not shown) are similar, so that the difference of the STD effect due to diversity of the T_1 relaxation time should be less important at least by using a saturation time of 2 s.[23] The STD effect of the bridgehead atoms can be affected by the near-by water signal in that way, that the integration gives a higher value than real, but we are optimistic to have eliminated this by the modified STD-pulse sequence and with a larger number of scans. It should be noted also, that the proton 5a shows some line broadening in every

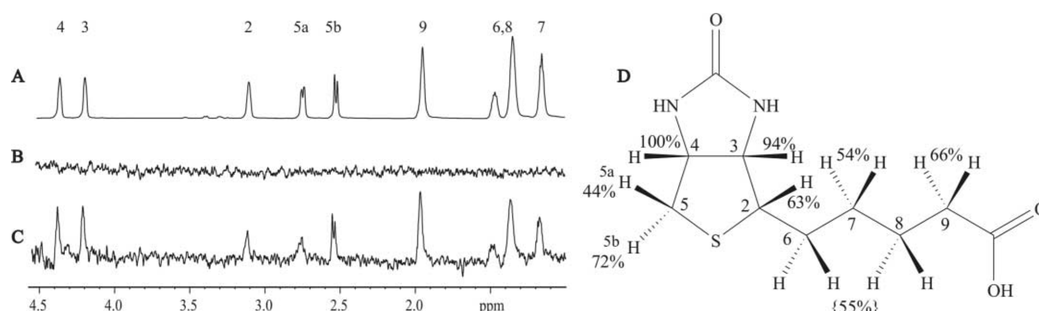


Fig. 1 All spectra were recorded at 278 K. A) The reference spectrum of 5 mM biotin and 0.05 mM 12S subunit. B) The STD-NMR control spectrum of 5 mM biotin without the protein, showing no signals. C) The corresponding STD-NMR spectra of 5 mM biotin and 0.05 mM 12S subunit recorded with 12 k transients displays that biotin signals appeared and therefore binds to the 12S subunit. D) Structure of biotin and the relative degree of saturation of individual proteins. The proton H4 was set to 100%. The protons 6 and 8 are overlapped and were treated as one group.

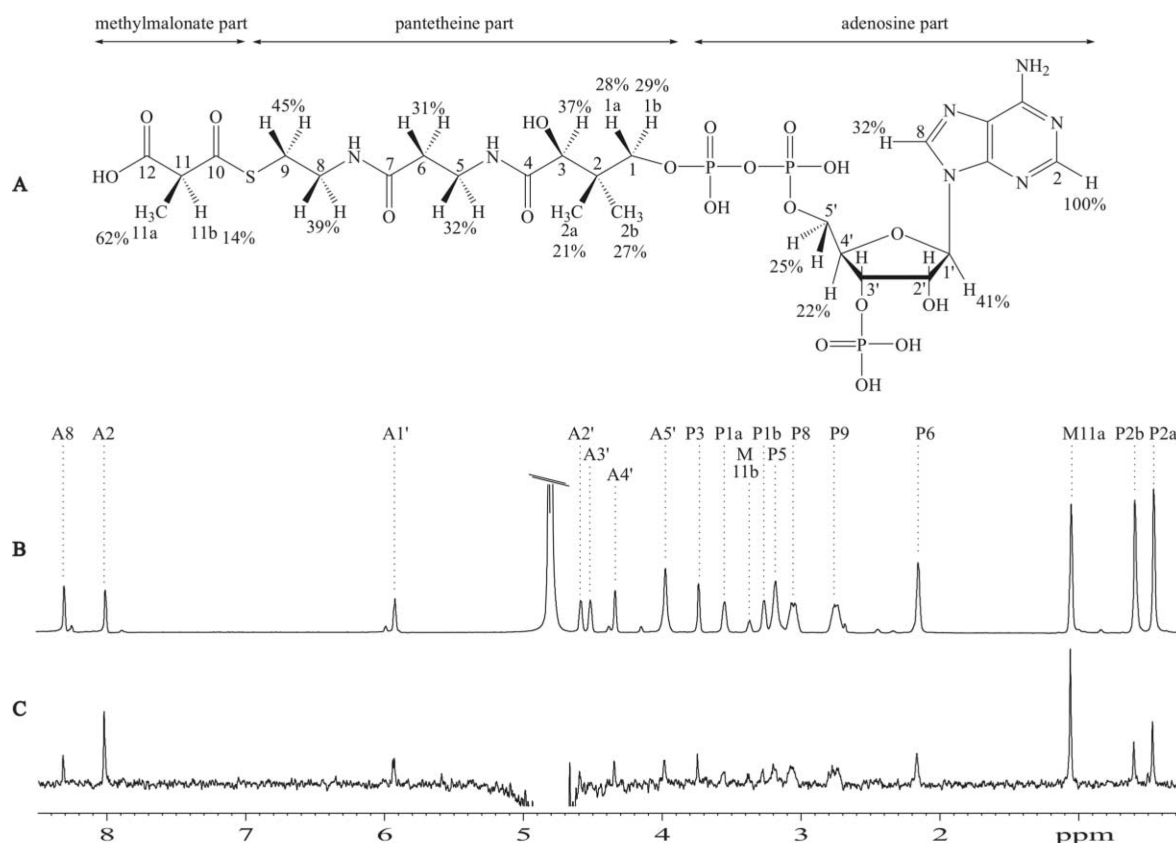


Fig. 2 A) Structure of MMCoA and the relative STD signal intensities obtained from C. The proton A2 was set to 100%. B) Reference spectrum of 2 mM MMCoA and 0.05 mM 12S subunit. C) Corresponding STD-NMR spectrum recorded with 12 k transients. Both spectra were recorded at 278 K.

STD-NMR spectrum, which is not explained at present. Nevertheless one can conclude that the bridgehead atoms show the highest contact to the protein and the protons of the valeryl side chain are not so close to the protein. This means that the ureido ring system is enclosed by the protein, which would make sense for the transfer of carbon dioxide to N1′. At the same time, the protein pocket might also reach the proton 5b. The STD effect generated from the 12S subunit to biotin is very low in comparison to MMCoA (see below), thus biotin is only a weak binder to the 12S subunit.

Epitope mapping of MMCoA to the 12S subunit of the TC

From MMCoA it is known that it binds to the 12S subunit. There are two different binding motifs, whereby the C-terminus of the 12S is more preferred, so that MMCoA lays in the interface between two opposing monomers.[16] But there is no information about the binding epitope of MMCoA. Fig. 2 displays a reference spectrum (B) and a STD-NMR spectrum (C) of 2 mM MMCoA and 0.05 mM 12S subunit at 278 K. The proton A2, with the largest integration value, was set to 100%. The relative integration value of the individual protons is shown in Fig. 2A. The adenine ring with the protons A2 (100% saturation) and A8 (32% saturation) show the highest effect. The malonate part with the proton M11 14% and the methyl group with 62% have also a high saturation. The difference of these two groups is attributed to the exchange processes of the proton M11 because of its acidic nature. The STD intensities are diminished along the pantetheine chain starting from the methylmalonate part. The protons P9 have 45% and the protons P8 have 39% saturation. The protons P6, P5, P3, P2 and P1 show similar STD effects ranging between 37% to 21%. The STD effect is also rather small in the ribose moiety of the adenosine moiety. The protons A1′ reach STD values of 41% and the protons A4′ and A5′ of about 25%. Also in this case one has to interpret the results with caution. The T_1 relaxation times (Table 1) of MMCoA have been determined in the presence and absence of the 12S subunit. The T_1 relaxation times in

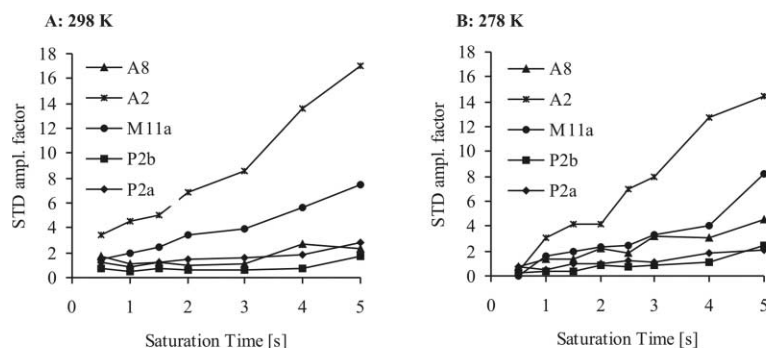


Fig. 3 Observed STD amplification factors of the adenine protons A8 and A2 and the methyl groups M11a, P2b and P2a of MMCoA plotted against different saturation times. The spectra were recorded with 2 mM MMCoA and 0.05 mM 12S subunit at 298 K (A) and at 278 K (B) and 3 k transients.

Table 1 The T_1 relaxation times of 2 mM MMCoA in the presence and absence of 0.05 mM 12S subunit obtained by the inversion recovery method at 278 K

MMCoA	T_1/s in presence of the 12S subunit	T_1/s without the 12S subunit
A8	1.73	2.04
A2	2.53	3.45
A1′	3.16	3.18
A4′	1.93	2.04
A5′	1.96	2.04
P3	1.04	1.16
P1a	1.82	2.06
M11b	0.84	0.94
P1b	0.77	0.87
P5	0.85	0.92
P8	0.76	0.62
P9	0.77	0.80
P6	0.81	0.90
M11a	0.74	0.88
P2b	0.43	0.47
P2a	0.53	0.59

the presence of the 12S subunit are a weighted average between the free and the bound state. Therefore the experimental T_1 relaxation times of MMCoA are nearly always larger in the absence of the 12S protein. Furthermore, the variations within the T_1 times are smaller in the presence of the protein, as expected. For example the proton A2 has a T_1 of 3.44 s and the methyl group M11 has a T_1 of 0.87 s for the free MMCoA. The same groups have T_1 relaxation times in the presence of the protein of 2.53 s for A2 and 0.74 s for M11. In order to exclude that the differences of the STD effect are not caused by the T_1 relaxation, STD-NMR spectra with different saturation times were recorded. For instance the proton A2 has a STD effect of 100% and the methyl group M11 of 62%. For a better assessment of the STD effects the STD amplification factor η as given in eqn. 1 was introduced:[17]

$$\eta = \frac{I_0 - I_{sat}}{I_0} \times \text{ligand excess} \quad (1)$$

This is the ratio of the intensities of the difference ($I_0 - I_{sat}$) and the reference spectra multiplied by the ligand excess, where I_0 is the intensity of the off-resonance signal. Fig. 3 shows the STD amplification factors for the adenine protons A2 and A8 and the methyl groups M11 and P2 of 2 mM MMCoA at 278 K and 298 K for different saturation times. It is shown, that the differences in the STD effect between the various protons observed are present already at the beginning of the built up curve and lasting throughout to the longest saturation time for both temperatures. The curves for the proton A8 and the methyl groups P2 are very similar despite the variation of the T_1 relaxation times for A8 with 1.72 s and P2b and P2a with 0.431 s and 0.532 s respectively. One can therefore conclude that the STD effects are not much influenced by the T_1 relaxation although the proton A2 with the largest saturation has a large T_1 time. Yan *et al.* have found that aromatic protons have higher STD intensities than aliphatic protons.[23] A detailed numerical calculation as shown by Bodenhausen *et al.*[24] was outside the scope of this work.

In summary, we observed that the adenine ring and the methyl group M11 from the methylmalonate moiety show the highest contact. On the pantetheine part the close contact is diminished. This is a good agreement with the published X-ray structure of the 12S subunit with MMCoA.[16] The 12S subunit shows the adenine recognition motif.[16,25] The proton A2 is enclosed by the protein, as the proton A8 is directed towards the ribose moiety, giving not a high contact. This result is supported by the proton A1′, having also a lower contact. The oxyanion hole formed by the 12S subunit is also presented in the epitope map.[26,27] The methyl group M11 and the beginning of the pantetheine chain give a close contact to the protein. This means that the carboxy group, the carbonyl group of the thioester and the sulphur itself interacts with the protein.

Competition binding experiments

For the competition binding experiments a solution of 5 mM Biotin and 0.08 mM 12S protein were titrated with MMCoA at 298 K, whereas after every addition a STD-NMR spectrum was recorded. Fig. 4 exhibits the STD curves of biotin and MMCoA during the titration of MMCoA. The STD amplification factor for the signals of biotin 6, 8 and 7 (A) and for the methyl groups M11 and P2 of MMCoA (B) is shown *versus* the increasing concentration of MMCoA. In Fig. 4C the averaged curves for all the protons of biotin and MMCoA are shown together. It stands out, that with an increasing concentration of MMCoA, the STD effect of MMCoA is increasing throughout. The STD effect for biotin is first increasing and then decreasing. The STD amplification factor of all biotin signals is increased to about 1.0–1.5 mM concentration of MMCoA. While further increasing concentration of MMCoA, the STD effect of biotin is diminished. The STD effect of biotin in the presence of MMCoA is larger than in the absence of MMCoA, this is attributed to a conformational change of the 12S subunit. This is in good agreement with Zheng *et al.*[28] They could observe by X-ray crystallography, the conformational change of the 12S subunit by adding MMCoA. The 12S subunit changes the conformation to shape the MMCoA binding pocket in order to bind MMCoA. Biotin shows a binding contact without the induced conformational change of MMCoA and a higher binding contact with that change. There is no significant difference in the biotin epitope mapping to the 12S subunit in the presence or absence of MMCoA (data not shown). It is of interest to raise the question, whether biotin alone is able to induce a conformational change of the 12S subunit to form a biotin binding pocket. This is, however, unlikely, on the basis of our

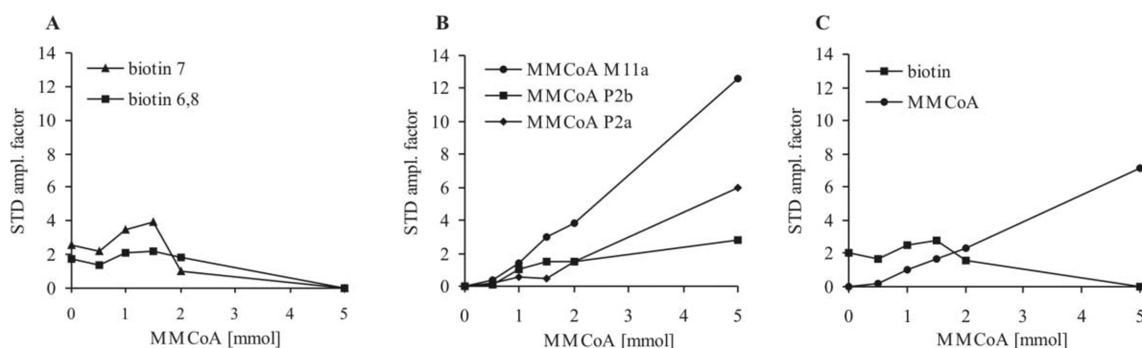


Fig. 4 Titration plot of 5 mM biotin and 0.08 mM 12S subunit with MMCoA at 298 K. After every addition step of MMCoA a STD-NMR spectrum with 3 k transients was recorded. The STD amplification factor is plotted against the concentration of MMCoA. A) The STD amplification factor of the signals biotin 7 and biotin 6,8 of biotin is shown. Noticeable is the increased STD effect until 1–2 mM concentration of MMCoA and with further addition of MMCoA the STD effect is decreased. B) The STD amplification factor of the methyl groups M11a, P2b and P2a of MMCoA increases with an increased concentration of MMCoA. C) The average STD amplification factor of biotin and MMCoA calculated from the data of A and B.

competition experiment. Biotin binds to the 12S subunit, but best at a 1.0 to 1.5 mM concentration of MMCoA using a concentration of the 12S protein of 0.08 mM. This corresponds to a ligand excess of MMCoA of about 12 to 18 fold over the 12S protein where biotin shows the best contact, but with even higher concentration of MMCoA, biotin is displaced by MMCoA. It is known that MMCoA binds to the 12S subunit and induces the conformational change. This happens if even two molecules of MMCoA bind to the 12S subunit.[28] The hexameric 12S subunit has 12 CoA ester sites, where MMCoA binds predominantly to domain 1; domain 2 appears to be unfavourable due to the potential steric hindrances.[16] Our results favour that biotin shows the best contact to the 12S subunit if all possible CoA ester sites are occupied. It seems that biotin binds to the same binding pocket as MMCoA, because on further additions of MMCoA, the STD effect of biotin is diminished. This means that the transfer of carbon dioxide from MMCoA to biotin happens cooperatively in one binding pocket, and best when MMCoA binds to all possible binding sites.

Conclusions

STD NMR measurements were used to prove interactions with *free* biotin and the carboxyl transferase subunit (12S) of the TC. It was shown that biotin binds to the 12S subunit, whereby the ureido ring system of biotin gives the highest contact. The group epitope mapping of MMCoA supports the results obtained by X-ray crystallography. There is a strong interaction of the 12S subunit with the adenine ring and the methylmalonate part of MMCoA. Competition binding experiments showed that biotin and MMCoA have one common binding pocket as biotin is a weaker binder than MMCoA. Biotin shows the highest contact, when all possible MMCoA binding sites were occupied. So it seems that carboxylation of biotin takes place cooperatively in one binding pocket. These findings will lead to a further investigation, where we want to carboxylate ^{15}N labelled biotin with ^{13}C labelled MMCoA in the presence or absence of the 1.3S subunit in order to test the functioning of enzymatic reaction without the tcc subunit forming one part of the natural TC complex.

Acknowledgements

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