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Heterologous expression of a deuterated membrane-integrated receptor and partial deuteration in methylotrophic yeasts

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Abstract

Methylotrophic yeast has previously been shown to be an excellent system for the cost-effective production of perdeuterated biomass and for the heterologous expression of membrane receptors. A protocol for the expression of 85% deuterated, functional human μ-opiate receptor was established. For partially deuterated biomass, deuteration level and distribution were determined for fatty acids, amino acids and carbohydrates. It was shown that prior to biosynthesis of lipids and amino acids (and of carbohydrates, to a lower extent), exchange occurs between water and methanol hydrogen atoms, so that 80%–90% randomly deuterated biomass and over-expressed proteins may be obtained using only deuterated water.

Introduction

Stable isotope labelling has become an essential tool in NMR spectroscopy of biomolecules (Kigawa et al., 1995; Lee et al., 1995; Venters et al., 1995; Gardner and Kay, 1998). ^{15}N and/or ^{13}C labelling is routinely used for the assignment, and determination of the structure and dynamics of large proteins and oligonucleotides. Deuterium labelling (LeMaster, 1989, 1990a,b) offers several specific advantages: uniform labelling of very large proteins reduces the ^{1}H, and ^{13}C linewidths and extends the applicability of 3D and 4D triple resonance experiments (Kushlan and LeMaster, 1993; Garrett et al., 1997; Kay and Gardner, 1997); selective deuteration may be useful in assignment (Markley et al., 1968; Putter et al., 1970; LeMaster, 1987; Oda et al., 1992; Reisman et al., 1993; Wishart et al., 1993; Curley et al., 1994; Kim et al., 1995; Metzler et al., 1996; Smith et al., 1996; Huang et al., 1997) and the selective protonation of amino acids allows the observation of numerous NOEs for more accurate structure determination (Oda et al., 1992; Metzler et al., 1996; Rosen et al., 1996; Smith et al., 1996; Gardner et al., 1997). By limiting spin diffusion, complete or partial deuteration makes it possible to get better NOE build up curves (Pachter et al., 1992), and is often required for the analysis of complexes between ligands and membranes or large proteins (Milon et al., 1990; Lee et al., 1991; Bersch et al., 1993; Anglister et al., 1995; Farmer et al., 1996; Fejzo et al., 1996; Constantine et al., 1997; Smith et al., 1997). Uniform and specific deuteration are also useful for deuterium solid state NMR analyses of protein – membrane interactions (Dahlquist et al., 1977; Davis, 1983; Prosser et al., 1994; Augé et al., 1995; Marsan et al., 1996a,b; Auger, 1997; Areas et al., 1998; Grobner et al., 1998), and neutron scattering experiments (Vanatalu et al., 1993; Shu et al., 1996; Reat et al., 1998).

Methylotrophic yeasts are a very attractive system for the production of both deuterated biomass and the heterologous expression of deuterated proteins (Tschopp and Cregg, 1991; Talmont et al., 1996; Hollenberg and Gellissen, 1997; Sreekrishna et al., 1997; Gallet et al., 1998; Phongdara et al., 1998), including

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$^{15}N/^{13}C$ labelled proteins (Laroche et al., 1994). Besides the classical advantages of yeasts, i.e. eukaryotic expression system, high growth rates and the obtention of extremely high cell densities, methylotrophic yeasts can be grown on very simple culture medium, containing salts and methanol as a unique carbon source. This makes ^{2}H, ^{15}N and ^{13}C labelling easily applicable and cost-efficient. We have previously shown that two strains, *Pichia angusta* and *Pichia pastoris*, can be adapted to growth in a fully deuterated environment, and were thus able to produce 16 g/l of biomass deuterated at 97.5% (Haon et al., 1993). From this biomass, perdeuterated lipids have been purified and characterised by NMR (Massou et al., 1998), thus providing perdeuterated model membranes directly usable for NMR characterisation of membrane-bound conformations of peptides. The growth rate was shown to be a function of the D/H ratio in the medium, being constant between 0 and 75% of deuteration ($\mu = 0.13\ h^{-1}$), and falling to $\mu = 0.02\ h^{-1}$ for 97.5% deuteration. This reduced growth rate was responsible for a marked reduction in the maximum biomass attainable (from 140 g/l to 16 g/l) and the efficiency of methanol conversion into biomass (from 30% to less than 5%, w/w), since at lower growth rates, much of the methanol is used for basal yeast metabolism. Since partial labelling is required in many of the experiments cited above, methylotrophic yeasts should be a very efficient tool for the production of biomass and proteins labelled in the 70% to 80% range, where growth occurs at a nearly maximum rate.

In the present paper a protocol for the heterologous expression of a 85% deuterated human μ-opiate receptor in *Pichia pastoris* has been established and various aspects relating to partial labelling have been addressed, e.g., (i) does the source of labelling (water or methanol) have an influence on the extent of deuteration?, (ii) does one get uniform labelling or specific enrichment of certain biomolecules, or specific sites such as the alpha/beta position of the amino acids?

Materials and methods

Growth conditions and culture media

All chemicals were obtained from usual commercial sources and used without further purification. Deuterated molecules (D_2O 99.8%, CD_3OD 99.8%) were obtained from Isotec, Inc.

Two strains were used throughout this study, *Pichia angusta* CBS 4732 and *Pichia pastoris* CBS 704. *Pichia angusta* cells were grown in three different culture media, M_a, M_b, et M_x(M_1 to M_4). M_a is a solid medium composed of YPD (yeast extract 1%; bacto-peptone 2%; glucose 2%) and agar 1.5%. The M_b liquid medium is composed of bacto-peptone 6% in distilled water, potassium phosphate buffer 0.1 M in D_2O, pH = 6.8, and methanol 5 g/l. The M_x liquid medium is composed of: KH_2PO_4 0.3% (weight%), $MgSO_4 \cdot 7H_2O$ 0.02%, $(NH_4)_2SO_4$ 0.15%, trace elements solution 2 ml/l (EDTA 5%, $ZnSO_4 \cdot 7H_2O$ 2.2%, $CaCl_2 \cdot 6H_2O$ 0.55%, $MnSO_4 \cdot 6H_2O$ 0.66%, $FeSO_4 \cdot 6H_2O$ 0.5%, $(NH_4)_2Mo_2O_7$ 0.11%, $CuSO_4 \cdot 5H_2O$ 0.15% and $CoCl_2 \cdot 6H_2O$ 0.16% in H_2O), vitamin solution 1 ml/l (biotin 0.4%, thiamine 3%), methanol 5 g/l, pH = 6.8. M_1 to M_4 differ in the deuterium labelling of water and methanol: $M_1 = H_2O/CH_3OH$, M_2 = 25% H_2O/75% D_2O/CH_3OH, $M_3 = D_2O/CH_3OH$, $M_4 = H_2O/CD_3OD$ (where D_2O and CD_3OD are 99.8% deuterated).

Cultures: Lyophilized yeast cells were resuspended in distilled water and placed on solid M_a rich medium. After 72 h at 30 °C, one clone was transferred into 2 ml of M_b medium and incubated for 72 h at 30 °C under agitation. Once growth was well established, 6.6×10^8 cell/ml, yeast cells were centrifuged (20 s, 13000g) and transferred aseptically at 6×10^6 cell/ml into the appropriate M_x medium. Growth was then allowed in Erlem meyer flask, at 30 °C up to a cell density of 2×10^8 cell/ml.

Analyses of deuterium labelling of carbohydrates, lipids and amino acids

Cell disruption: *Pichia angusta* yeast cells were harvested by centrifugation and broken in the presence of equal amounts of 450–600 μm glass beads (Sigma) and water by 40 min vortexing. After addition of 1 ml of water, the mixture was centrifuged at 17000g for 10 min to remove intact cells, then at 100 000g for 1 h to separate membranes and cell wall fragments (pellet) from cytosolic proteins (supernatant). A BioRad protein assay showed that the supernatant was mainly composed of proteins.

Carbohydrates: Cell wall fragments were hydrolysed: 2 mg of cell walls were dissolved in TFA 2M (100 μl) and kept for 2 h at 110 °C in a sealed tube and the hydrolysis products were dried under a nitrogen stream. The monosaccharides were then transformed into trimethyl silyl derivatives as in DeJongh et al. (1969).

Lipids: the lipids were extracted, saponified, and the fatty acids esterified as described by Tropis et al. (1996).

Amino acids: Two mg of cytosolic proteins or total membrane proteins was hydrolysed in 6N HCl ('constant boiling', Sigma) or ^{2}HCl (Isotec, Inc.) during 12 h under reflux according to standard procedures. Solvent was subsequently removed under a nitrogen stream. *N*-Perfluoropropyl,n-butyl ester derivatives was then prepared: 200 µl of hydrogen chloride in butanol were added to 1 mg of the amino acid mixture and incubated for 30 min at 110 °C under argon atmosphere. After evaporation under a nitrogen stream, 100 µl of perfluoropropionic anhydride (Pierce, Inc.) was added and left for 30 min at 110 °C under argon atmosphere.

GC analyses of TMS monosaccharides, fatty acid methyl esters and *N*-perfluoropropyl,n-butyl ester amino acids were performed on an OV1 WCOT column (25 m × 0.22 mm) programmed from 50 to 280 °C (3 °C/min). GC/MS spectra were acquired on an HP-1 glass column, programmed from 50 to 290 °C (8 °C/min) and interfaced to a Hewlett-Packard 5989A mass spectrometer. Fatty acid derivatives were analysed by EI/MS (ion source temperature 250 °C, ionisation voltage 70 eV). TMS monosaccharides were analysed by CI/MS with NH_3 as a reagent gas (ion source 250 °C, ionisation voltage 230 eV). Deuteration extents were calculated from GC/MS spectra on molecular ions for fatty acid methyl esters, specific fragment for amino acid derivatives and $(M + NH_4)^+$ molecular ions for monosaccharide derivatives. Since each ion gave a complex spectrum, the isotopic distribution was analysed according to McCloskey (1990), after correction for contributions from carbon and silicon isotopes, in order to extract the average deuterium labelling (probability of deuteration on each protonated site). Briefly, for an ion of molecular weight M containing W exchangeable hydrogens, with a deuteration probability on each site equal to *p*, P(X), the intensity of peak M + X (X sites deuterated) is given by $P(X+1)/P(X) = (W-X).p/(X+1).(1-p)$, if the labelling is uniform and statistically distributed. This distribution, illustrated in Figure 1, depends both on W and on *p* and can be used to determine both parameters. The determination of W allows to discriminate between uniform or site-specific labelling (for which W is smaller than the total number of hydrogens).

For NMR analyses of amino acids, 10 mg of protein was hydrolysed (HCl 6N, 110 °C, 12 h) and the solution centrifuged (13000g, 2 min). The supernatant composed of amino acids was dried under nitrogen and dissolved in a 0.2 M potassium phosphate buffer in D_20, pH = 7.4 (uncorrected for isotopic effect). All the NMR experiments were performed at 298 K, on a BRUKER AMX500 spectrometer. 2D DQF-COSY gradient enhanced spectra were acquired using a gradient ratio 1:2 (7:14 Gauss/cm) to select for double quantum coherences. Sinusoidal shaped gradients with a 5 ms length and a recovery delay of 200 µs were used. The spectral width was 2320 Hz in both dimensions. The acquisition time was 880 ms and the relaxation delay was 1 s. The number of accumulations per t_1 experiment was 4 and the number of t_1 experiments was 1024. The data were zero filled in both dimensions. The processing was performed with sine-bell window functions in both dimensions.

Expression of the human µ-opiate receptor and binding experiments

Pichia pastoris cells transformed for the expression of human µ-opiate receptor (Talmont et al., 1996) were first grown for 96 h at 30 °C on the following medium: Yeast Nitrogen Base without amino acids (DIFCO, 1.34 g in 100 ml D_2O), 1 M potassium buffer, pH = 7.5, in D_2O, 10% glycerol in D_2O, in a 8/1/1 volume ratio. After production of sufficient biomass, cells were centrifuged and resuspended in the same volume of 'inducing' medium (same as above, except for the 10% glycerol solution in D_2O which was replaced by a 10% methanol solution in D_2O, to enhance the AOX promoter) and further grown for 15 h at 30 °C for the production of receptor. Membranes expressing the receptor were recovered after disruption of yeast cells and differential ultracentrifugation. Membrane proteins were analysed for their deuteration content as described above. [^{3}H]-Diprenorphine binding assay and total binding sites were measured on whole cells as described previously (Talmont et al., 1996).

Results and discussion

Labelling extent in lipids, proteins and carbohydrates

In order to determine the extent of hydrogen exchange between water and the carbon source, methanol, *Pichia angusta* cells were grown in media containing either deuterated water and hydrogenated

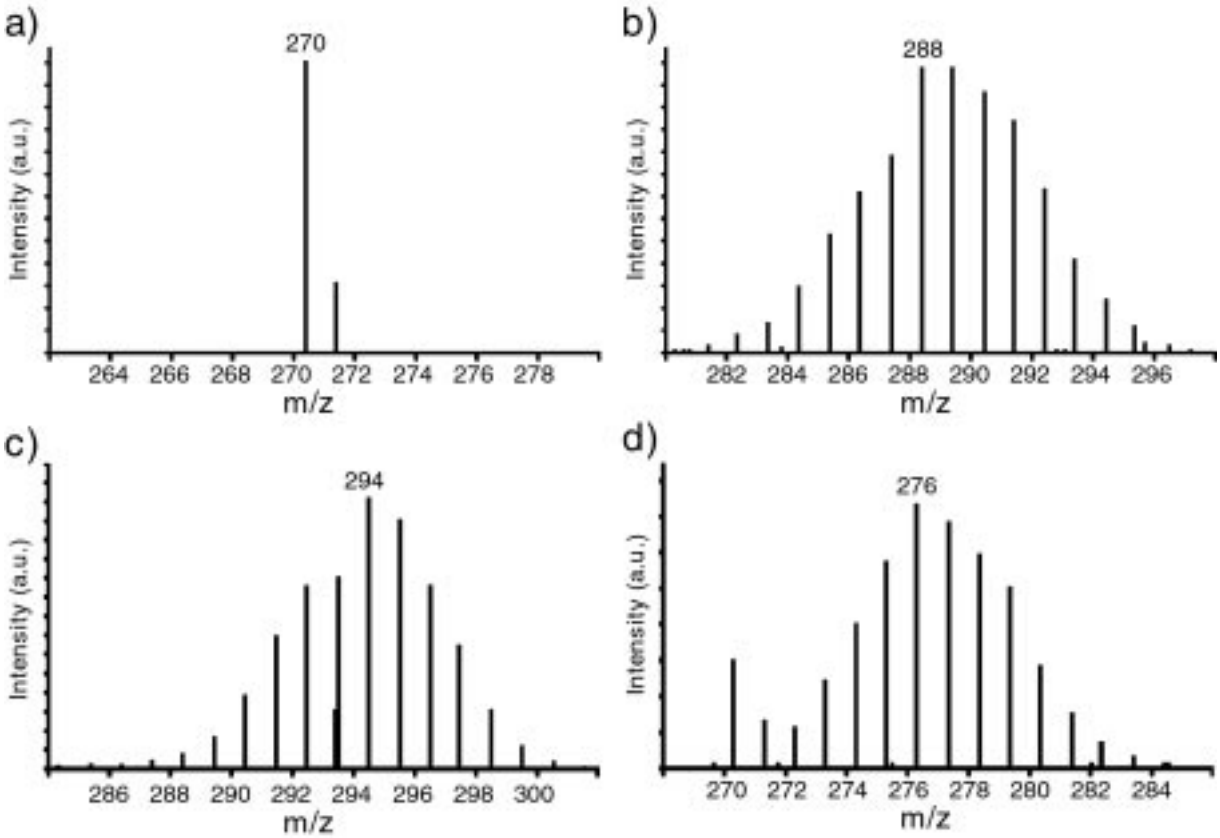

Figure 1. GC-MS spectra: molecular ions of palmitoyl methyl ester isolated from *Pichia angusta* grown on (a) M_1 medium, H_2O/CH_3OH; (b) M_2 medium, 75% D_2O/CH_3OH; (c) M_3 medium, D_2O/CH_3OH; (d) M_4 medium, H_2O/CD_3OD. Statistical analysis of the peak distribution gives the following deuteration levels: (a) 0%; (b) 62%; (c) 80%; (d) 22%.

methanol (M_2,M_3) or hydrogenated water and deuterated methanol (M_4). After biomass production, the deuteration extent in three major classes of metabolites, amino acids, fatty acids and carbohydrates was determined. Figure 1 shows the molecular ions of palmitoyl methyl ester after growth on differently labelled media. Analysis of the isotopic distribution shows that the labelling is uniform (W equal to the total number of hydrogens) and leads to an average deuteration probability on each site, *p*, equal to 62% for M_2, 80% for M_3 and 22% for M_4. This is to be compared with the expected deuteration levels if the biomass hydrogen atoms were arising exclusively from the carbon source (0% for M_2, 0% for M_3 and 100% for M_4), and if, at the other extreme, complete exchange were occurring between water and methanol hydrogen atoms (75% for M_2, 99% for M_3 and 0.5% for M_4). This clearly demonstrates that some limited exchange occurs, and that statistically, in all cases, 80% of the hydrogen atoms incorporated into the fatty acids are coming from water, and 20% are from methanol. However, since the deuterium enrichment on M_4 medium (22%) is 40 times higher than the average D/H ratio in the growth medium (0.5%), a high specificity for methanol hydrogen atoms was observed.

In order to get some indications as to where this proton exchange occurred, the deuteration level and distribution on the amino acids and carbohydrates was further analysed. Deuteration levels of 12 amino acids were monitored by GC/MS analysis of *N*-pentafluoropropyl,n-butyl ester derivatives (Kaiser et al., 1974; Demange et al., 1988) after hydrolysis of cytosolic proteins (Figure 2a). The molecular ion intensity being too weak, deuteration levels were determined from a fragment ion corresponding to the loss of carboxylate, which contains all the potentially deuterated sites (Figure 2b). For the amino acids which were not separated by GC, Asp/Phe and Glu/Tyr, more specific fragmentations did allow the analysis without

Table 1. Deuterium labelling (in %) of amino acids obtained from growth in M_3 (D_2O, CH_3OH) and M_4 (H_2O, CD_3OD) culture media

	Ala	Gly	Val	Thr	Ser	Leu	Ile	Pro	Phe	Asp[a]	Glu[a]	Tyr[a]
M_3 (D_2O)	88	78	80	82	85	85	81	85	80	42	70	65
M_4(CD_3OD)	15	33	14	25	20	18	11	18	31	14	10	nd

nd: not determined; [a] H/D exchange during protein hydrolysis explains the apparent lower labelling.

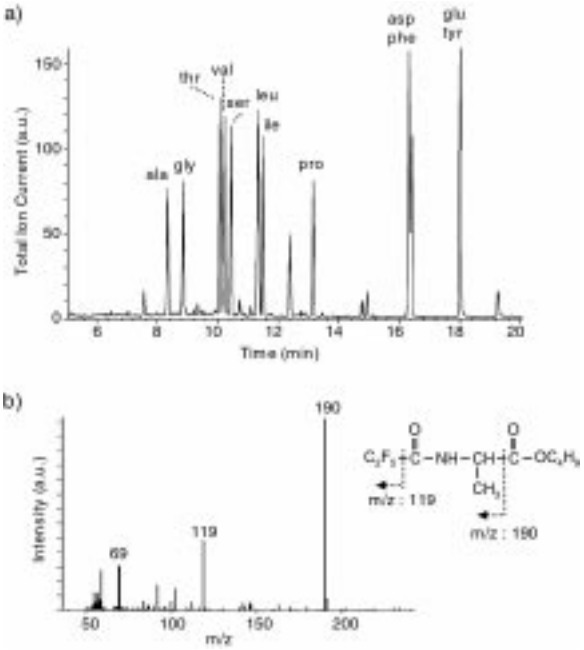

Figure 2. GC-MS analysis of amino acid mixtures. (a) Chromatogram of *N*-perfluoropropyl, n-butyl ester amino acid derivatives; (b) mass spectrum and major fragmentations of the unlabelled alanine derivative.

ambiguity. The results for cells grown in M_3 and M_4 media are shown in Table 1.

For the first 9 amino acids, the distribution was found to be uniform, and deuteration levels of 83 ± 5% and 20 ± 10% were found for M_3 and M_4 media, respectively. The differences between these is within the range of experimental error. These levels are very close to those found for fatty acids. The slightly different deuteration levels found for the last three amino acids were due to chemical exchange occurring during protein hydrolysis. It was confirmed by DCl hydrolysis that chemical H-D exchange is not significant in the alpha position of amino acids (<5%) (Rosen et al., 1996), but is efficient on Asp and Glu side chains (position alpha to carboxylates), and on the tyrosine phenol (positions *ortho* to the hydroxyl group).

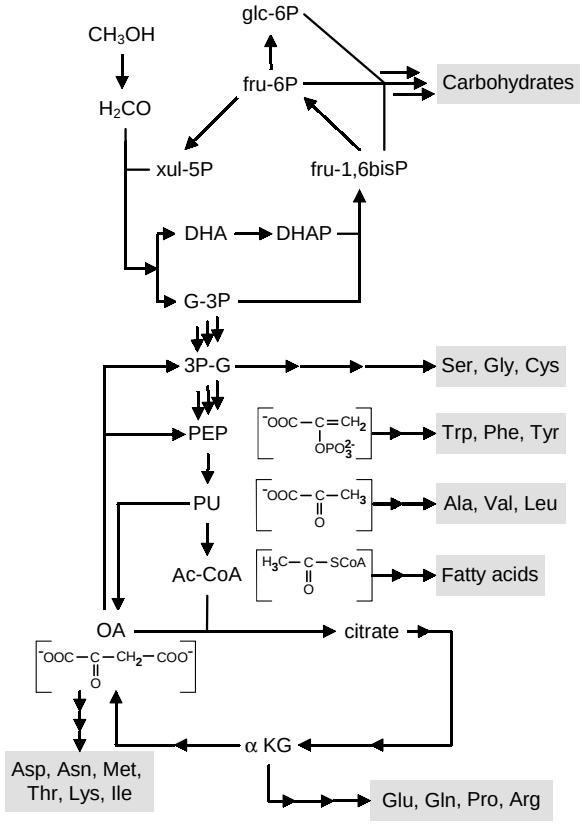

Figure 3. Simplified metabolic pathway for the production of amino acids, lipids and carbohydrates from methanol in methylotrophic yeasts. The chemical structures of the intermediates which may account for efficient H/D exchange are indicated (PEP, PU; Ac-CoA, OA). Abbreviations: CH_3OH, methanol; H_2CO, formaldehyde; xul-5P, xylulose-5-phosphate; fru, fructose; glc, glucose; DHA(P), dihydroxyacetone (phosphate); G-3P, glyceraldehyde-3-phosphate; 3P-G, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PU, pyruvate; Ac-CoA, acetyl CoA; OA, oxaloacetate; αKG, alpha ketoglutarate.

Cell wall carbohydrates were analysed according to standard procedure by GC-MS. They were composed of glucose and mannose in a glc/man ratio of 0.70 ± 0.03 independently of the M_x culture medium. The uniform deuteration extent was 60% for the M_3 medium and 40% for the M_4 medium. These numbers

are clearly different to those seen for lipids and amino acids, showing that hydrogen atoms belonging to the carbon source are more efficiently incorporated into carbohydrates than into lipids and amino acids.

This can be understood considering the simplified metabolic pathway for the biosynthesis of carbohydrates, lipids and proteins from methanol in the methylotrophic yeasts (Figure 3). Fatty acids and amino acids share common precursors such as pyruvate and acetyl CoA which may allow for chemical exchange with water protons by reversible enolisation. Other intermediates such as oxaloacetate in the Krebs cycle, or malonyl CoA for fatty acid biosynthesis, contain more acidic protons which could enable a more efficient chemical exchange. However, since the exchange level is identical for Phe, Ala, Val and Leu, whose biosynthesis does not involve these intermediates, one may assume that the exchange occurs mainly at the level of central metabolites. One plausible central point for hydrogen exchange is the reversible enzymatic interconversion between the three carbon acids, pyruvate, 3-phosphoglycerate and phosphoenolpyruvate, possibly involving also the futile C_3-compound to C_4-compound interconversion cycle by anaplerotic reactions necessary for NADPH generation in certain micro-organisms (Dominguez et al., 1998). Carbohydrate biosynthesis in methylotrophic yeasts does not pass via these intermediates, and may be synthesised directly from sugar phosphates generated from the assimilation of methanol via the condensation reaction involving xylulose-5-phosphate and formaldehyde (Waites et al., 1981), thus explaining a reduced exchange with water protons.

One important consequence of this exchange mechanism is that partial deuteration is expected to be uniform, and statistically distributed in each protonated site. Indeed, the analysis of fragments by mass spectrometry clearly demonstrated that this was the case for all amino acids analysed, once experimental errors were taken into consideration, as well as for fatty acids and sugars. A control NMR experiment was performed in order to detect the existence of amino acid hydrogen atoms which would come *directly* from methanol, prior to the exchange mechanism at the C_3-compounds level discussed above. Biomass was produced using a culture medium containing H_2O and CD_3OD, so that direct incorporation from CD_3OD would lead to 100% deuteration in these conserved sites and to the complete disappearance of the corresponding cross peak in a 2D COSY experiment. Figure 4 shows ge-DQF-COSY spectra of amino acid mixtures obtained after growth in M_1 (H_2O, CH_3OH, no labelling) and M_4 (H_2O, CD_3OD, 20% labelling on average) media: although some differences were observed between both spectra, which might be due to differences in quantities and/or slightly different amino acid composition in both mixtures, most of the cross peaks found in the unlabelled sample were present in the labelled sample, with a uniformly reduced intensity for the labelled sample due to the average 20% deuteration. Particular attention was paid to the methyl cross peaks, because of the previously reported production of deuterated proteins being specifically protonated on some Ala, Val, Leu and Ile methyl groups, using deuterated water and protonated pyruvate (Rosen et al., 1996), and because of the possibility of stereospecific labelling of Val methyls due to the different metabolic pathways for the incorporation of the two prochiral methyl groups in Val and Leu amino acids (Neri et al., 1989). The enlargement of the H_β–$H_{\gamma 1}$, H_β–$H_{\gamma 2}$ Val cross peaks is shown in Figure 4, Ab and Bb. Again slight differences in the cross peak patterns are observed in the absence and presence of deuterated methanol, which can be explained by the 20% uniform deuteration in Figure 4, Bb. However, both methyls at 0.98 and 1.03 ppm are observed with similar intensities, excluding the hypothesis of a 100% deuteration of one of them due to a direct incorporation from CD_3OD. Therefore, this system does not lead to highly regio- or stereospecific labelling, at least under the growth conditions employed here, and offers a more uniform label incorporation than other systems described so far in the literature (Neri et al., 1989; Szyperski et al., 1992; Rosen et al., 1996; Gardner et al., 1997).

It may seem surprising to observe a statistical distribution of labelling, and yet 20% deuteration when 100% H_2O/100% CD_3OD is used, instead of 0.5% which would be expected for a complete exchange. The reason for this is probably that limited exchange occurs on a central C_3-compound such as pyruvate (CD_3-CO-COO^-) forming a CD_3, CD_2H, CDH_2, CH_3 mixture, from which all proton sites in the biomass will be formed, with the same deuteration extent as the parent methyl.

Production of deuterated human μ*-opioid receptor*

To exploit this system for biochemical studies, it was demonstrated that a methylotrophic yeast can be used to produce a highly deuterated, functional, membrane receptor. Deuterated membrane proteins are required for neutron scattering experiments (Reat et al., 1998),

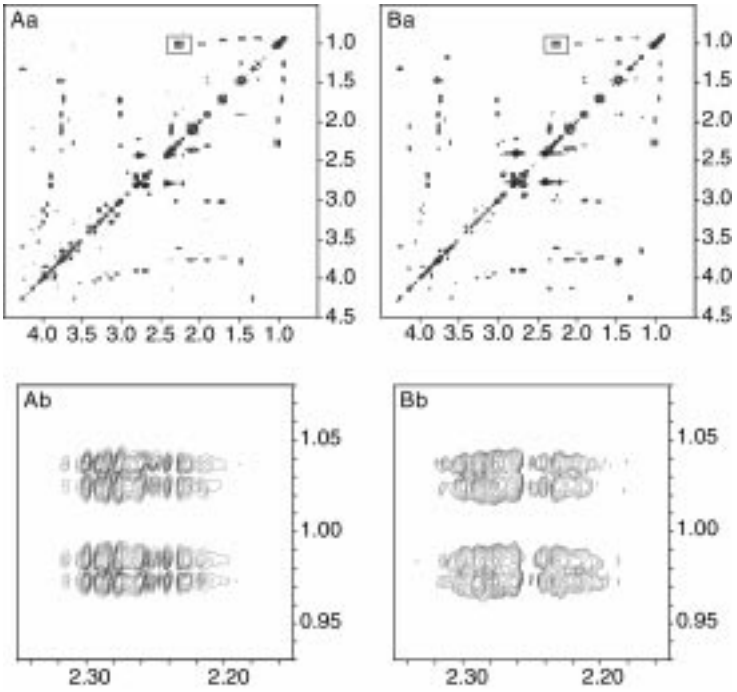

Figure 4. ge-DQF-COSY of an amino acid mixture prepared from *Pichia angusta* cells grown on (A) unlabelled medium; (B) M_4 medium (H_2O/CD_3OD), 20% labelling; (a) 0–4.7 ppm region; (b) enlargement of the valine H_β-$H_{\gamma 1}$, H_β-$H_{\gamma 2}$ cross peaks.

and for NMR. One major challenge in the field of opiate receptor transduction signal is the determination of the receptor-bound conformation of opioid neuropeptides (Befort et al., 1996; Blake et al., 1997). This can be performed using either transferred NOE for rapidly exchanging (e.g. low affinity binding) peptides (Milon et al., 1990; Bersch et al., 1993; Fejzo et al., 1996), or ^{15}N-isotope edited NMR on detergent-solubilised ligand–receptor complexes (Constantine et al., 1997; Ottleben et al., 1997), possibly using the recently developed TROSY experiment (Pervushin et al., 1997). Both types of experiment require highly deuterated receptor and lipids in order to reduce spin diffusion on these large molecular weight systems.

Pichia pastoris cells expressing the human μ-opioid receptor (Talmont et al., 1996) were first grown for 96 h at 30 °C on the YNB amino acid-free medium. After centrifugation and elimination of the medium, living cells were induced in a medium where glycerol was replaced with 10% methanol in D_2O to enhance the AOX promoter. The deuteration was then determined on total membrane proteins as described previously. In preliminary experiments using complete yeast extract, GC-MS analysis of amino acids indicated the presence of two populations: one properly deuterated, and one unlabelled, presumably due to a direct incorporation of amino acids from the medium. The use of YNB solved this problem, and the deuteration levels of amino acids were in the 85–90% range, using deuterated water and hydrogenated glycerol and methanol. This protocol therefore allows the large scale production of deuterated receptor at reasonable cost, since only the fairly cheap deuterated water is required.

Whole cells of the transformed yeast strain grown on D_2O exhibited specific saturable binding of [^{3}H]-diprenorphine with a $K_d = 0.54 \pm 0.09$ nM, a value comparable with the $K_d = 0.61 \pm 0.09$ nM determined for yeast grown on a fully hydrogenated medium. Growth rates, and expression levels were also identical in both cases, with about 200 ligand binding sites per cell. This expression level of deuterated receptor is sufficient to prepare membranes for transferred NOE experiments (Milon et al., 1990; Bersch et al., 1993) and to determine the receptor-bound conformation of neuropeptides. Such an experiment requires a 10 μm concentration of receptor (Czaplicki et al., 1998), i.e., 10 nmoles of purified receptor which can be prepared from a 10 l fermentation (assuming 200 receptors/cell, 3×10^{13} cell/l and a 10%

purification yield). Furthermore, the optimisation of growth and induction conditions in standard media has already led to higher expression levels (2000 sites per cell) which are required for larger scale expression of the receptor.

Conclusions

Methylotrophic yeasts are an excellent tool for the cost-efficient production of biomass uniformly deuterated to any level. For label incorporation up to 80% one need not use a deuterated carbon source, growth rates and attainable biomass are very high (normal growth rate and up to 100 g yeast biomass/l). Labelling up to 97.5% is possible, although at an increased cost. Another advantage for NMR is that these yeasts grow naturally and easily on a minimal medium, and that the carbon source is a C_1 molecule: randomly distributed ^{15}N and ^{13}C labelling to any level are therefore possible and can be combined with deuterium labelling. Indeed, 10 to 20% ^{13}C labelling is needed in solid state NMR of proteins in order for instance to measure H-C dipolar couplings on isolated spin pairs while reducing C-C strong dipolar coupling from adjacent carbons (Griffin, 1998). A similar strategy using a methylotrophic bacterium, *Methylophilus methylotrophus*, has been described (Batey et al., 1992, 1996) though this does not generate the same spectrum of biomolecules as an eukaryotic system. Methylotrophic yeasts possess also an excellent potential for the heterologous expression of a functional membrane receptor in an eukaryotic system, as shown here for the μ-opiate receptor, and for soluble proteins (Sudbery, 1996; Hollenberg and Gellissen, 1997; Gallet et al., 1998). They thereby present an ideal tool for ^{2}H, ^{15}N and ^{13}C labelling of membrane and soluble proteins, lipids and carbohydrates.

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