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Bioreaction Network Topology and Metabolic Flux Ratio Analysis by Biosynthetic Fractional ^{13}C Labeling and Two-Dimensional NMR Spectroscopy

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Biosynthetically directed fractional ^{13}C labeling of the proteinogenic amino acids is achieved by feeding a mixture of uniformly ^{13}C-labeled and unlabeled carbon source compounds into a bioreaction network. Analysis of the resulting labeling pattern enables both a comprehensive characterization of the network topology and the determination of metabolic flux ratios. Attractive features with regard to routine applications are (i) an inherently small demand for ^{13}C-labeled source compounds and (ii) the high sensitivity of two-dimensional [^{13}C,^{1}H]-correlation nuclear magnetic resonance spectroscopy for analysis of ^{13}C-labeling patterns. A user-friendly program, FCAL, is available to allow rapid data analysis. This novel approach, which recently also has been employed in conjunction with metabolic flux balancing to obtain reliable estimates of *in vivo* fluxes, enables efficient support of metabolic engineering and biotechnology process design. © 1999 Academic Press

Key Words: **amino acid biosynthesis; biosynthetic fractional ^{13}C labeling; central carbon metabolism; 2D NMR; *in vivo* flux ratios; metabolic flux balancing; METAFoR analysis.**

INTRODUCTION

Redirecting metabolic carbon and energy fluxes to achieve increased production, conversion, or degradation of biotechnological target compounds can be realized by optimizing bioprocess parameters and/or genetic manipulations (Bailey, 1991). The operation of bioreaction networks represents highly nonlinear processes of holistic nature, so that the success of reductionistic approaches to rationalize and predict metabolism have often been limited. Although recent theoretical advances, *e.g.*, in the framework of cybernetic modeling (Varner and Ramkrishna, 1999), have significantly extended predictive capabilities, we are far from understanding all principles governing metabolic regulation. The development of methods for experimental assessment of a bioreaction network's response to changes in growth conditions or genetic manipulations thus plays a pivotal role for bioprocess optimization. Methods for unravelling the network topology and the *in vivo* flux distribution are of primary interest: The topology defines the limits of the production capacity of a given network, and the fluxes represent a final balanced manifestation of all cellular components connected to metabolic regulation.

This article outlines a novel approach that allows both a comprehensive characterization of a network topology and the monitoring of metabolic flux distributions. It is based on biosynthetically directed fractional (BDF) ^{13}C labeling of the proteinogenic amino acids, which is achieved by feeding a mixture of uniformly ^{13}C-labeled ([U-^{13}C]-labeled) and unlabeled carbon source molecules into a bioreaction network (Senn *et al.*, 1989; Neri *et al.*, 1989; Wüthrich *et al.*, 1992; Szyperski *et al.*, 1992). When employed in conjunction with 2D [^{13}C,^{1}H]-correlation NMR spectroscopy for efficient analysis of the resulting ^{13}C-labeling pattern in the

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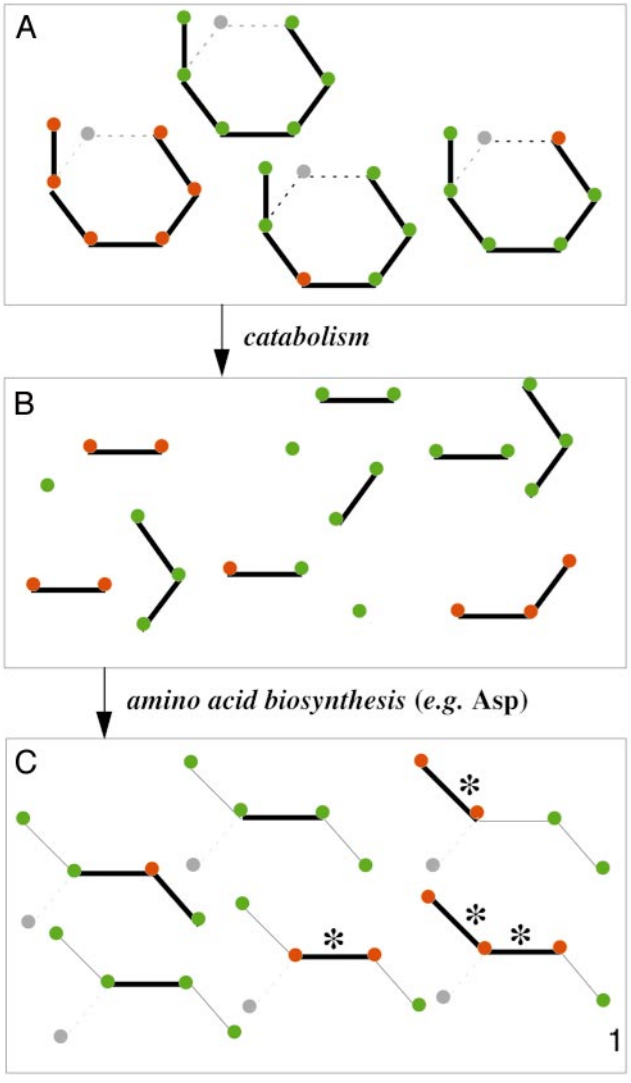

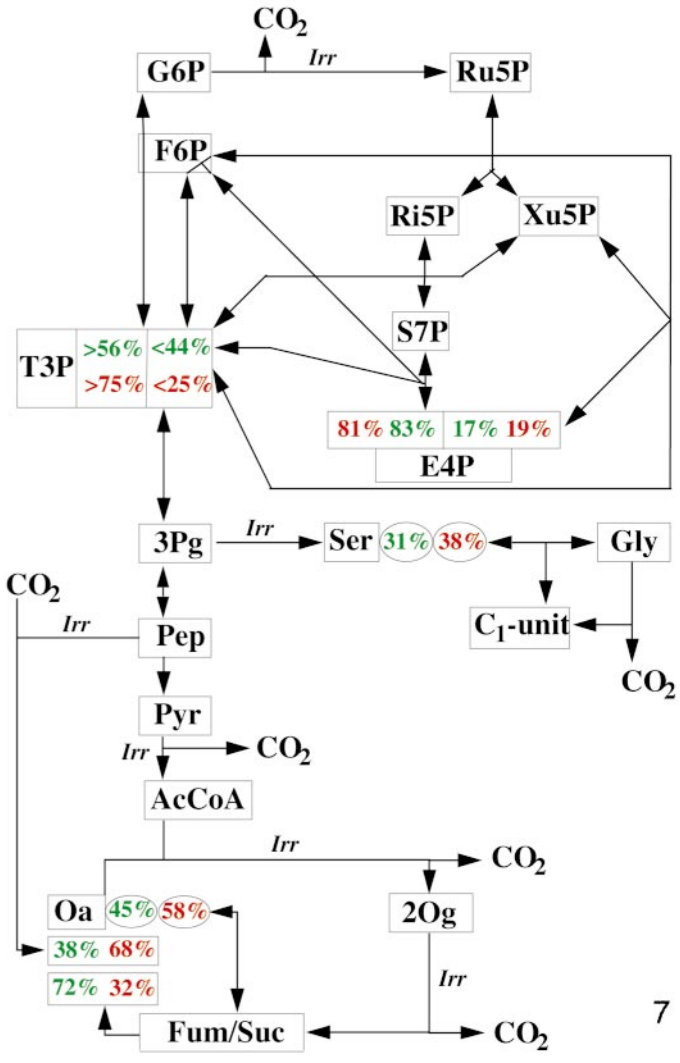

FIG. 1. Schematic presentation of the principle of biosynthetically directed fractional ^{13}C labeling achieved by growing cells in a minimal medium with 25% [U-^{13}C]-labeled glucose and 75% glucose containing ^{13}C at natural abundance as the sole carbon source (A). ^{13}C and ^{12}C nuclei are represented by red and green dots, respectively. In (B) and (C), carbon–carbon connectivities that were not cleaved due the action of metabolism are indicated by the same bold lines as the bonds of glucose in (A), while covalent bonds formed during amino acid biosynthesis are depicted with thin lines. Asp has been chosen as an example. Intact connectivities detectable by NMR (see text) are labeled with an asterisk. Oxygen and nitrogen atoms of glucose and aspartate are depicted as grey dots in (A) and (C), respectively. Since Asp is derived from oxaloacetate, such analysis allows one to quantitate the supply of the tricarboxylic acid cycle (Szyperski, 1995).

FIG. 7. METAFoR analysis of the tricarboxylic acid cycle, the pentose phosphate pathway, and C_1 metabolism of wild-type *E. coli* cells grown under carbon limitation (green numbers) or nitrogen limitation (red numbers) in chemostat cultures (Sauer *et al.*, 1999). Irreversible reactions are indicated by single-headed arrows and are denoted with "*Irr*," while mutual interconversions are represented by double-headed arrows. The fractions of molecules given in square boxes are synthesized *via* the fluxes pointing into them. The fractions displayed in ellipses indicate the extent of reversible interconversion of the molecule in question. Metabolic intermediates used for synthesis of the proteinogenic amino acids are shown in bold. Abbreviations: AcCoA, acetyl-CoA; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; Fum, fumarate; G6P, glucose 6-phosphate; Gly, glycine; Oa; oxaloacetate; 2Og, 2-oxoglutarate; 3Pg, 3-phosphoglycerate; Pep, phospho*enol*pyruvate; Pyr, pyruvate; Ri5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; S7P, seduheptulose 7-phosphate; Ser, serine; Suc, succinate; T3P, triose 3-phosphate, *i.e.*, glyceraldehyde 3-phosphate and dihydroxyacetone-phosphate; Xu5P, xylulose 5-phosphate.

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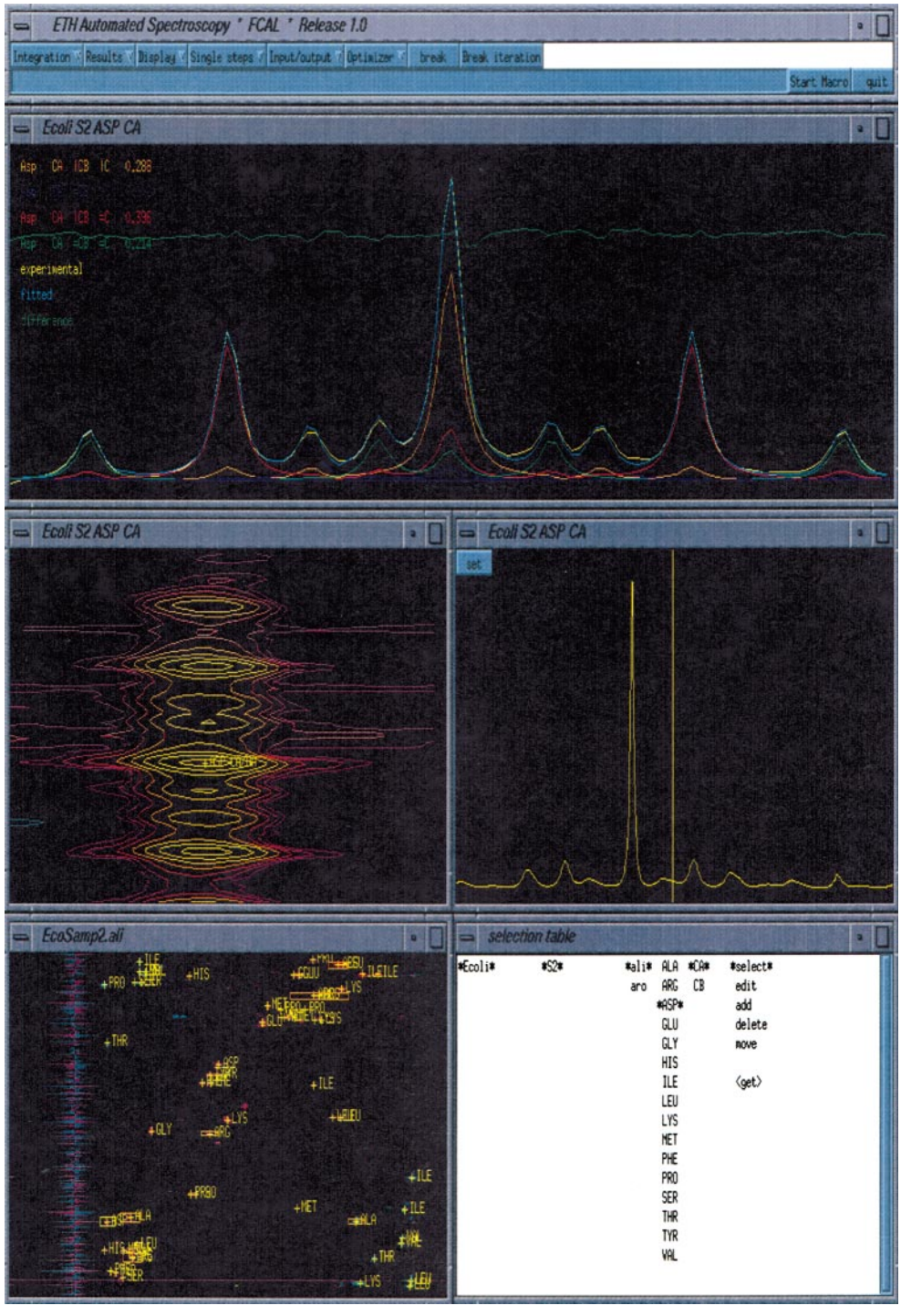

FIG. 5. User interface of the program FCAL. The control window at the top contains the pull-down menus and the command line to interactively run the program. The "fitting window" below displays a one-dimensional representation of the experimental (yellow) and the fitted (blue) ^{13}C–^{13}C scalar coupling fine structure, as well as the residual difference between the experimental and the fitted fine structure (green). The relative abundances of intact fragments inferred from this fit are also indicated. This window is repeatedly updated during the nonlinear least-squares fit. As an example, the fit obtained for $^{13}C^{\alpha}$ of Asp is shown (see Figs. 2B, 2C, and 3A). The two central windows display the corresponding spectral region taken from the 2D [^{13}C,^{1}H]-COSY spectrum (on the left) and the result of a grid search that may be performed to initially assess the ^{13}C chemical shift (on the right). The spectral overview window at the bottom on the left supports the interactive selection of individual peaks, and the text window (on the right) allows efficient bookkeeping when analyzing a large number of samples.

amino acids, BDF ^{13}C labeling yields a detailed picture of the breakdown of precursor molecules in the bioreaction network under consideration (Szyperski, 1995). This enables the identification of active biochemical pathways (Szyperski, 1995; Sauer *et al*., 1997; Hochuli *et al*., 1999), the determination of flux ratios, and the semi-quantitative assessment of exchange fluxes (Szyperski, 1995; Szyperski *et al*., 1996; Sauer *et al*., 1997; Szyperski, 1998; Fiaux *et al*., 1999; Sauer *et al*., 1999). Clearly, limitations of this approach occur in case two alternative pathways operate according to the same carbon–carbon bond rearrangements. For example, glycolysis and Entner–Doudoroff pathway generate equally ^{13}C-labeled C_3 units when feeding glucose as the sole carbon source, and may thus not be distinguished (Szyperski, 1995).

METHODS

Biosynthetically Directed Fractional (BDF) ^{13}C *Labeling: The Basic Principle*

To gain insight into the action of metabolism, [U-^{13}C] molecules and/or fragments thereof must be "diluted" with either nonenriched endogeneous pools, *i.e*., intracellular metabolites, or nonenriched exogeneous pools, *i.e*., carbon source molecules provided in the growth medium (Szyperski, 1998). This allows one to trace conserved ^{13}C–^{13}C connectivities in the metabolic network. In particular, BDF ^{13}C labeling employed with a minimal medium containing a mixture of [U-^{13}C] and unlabeled carbon source molecules ensures that the metabolic dilution of [U-^{13}C] fragments is entirely determined by the composi-

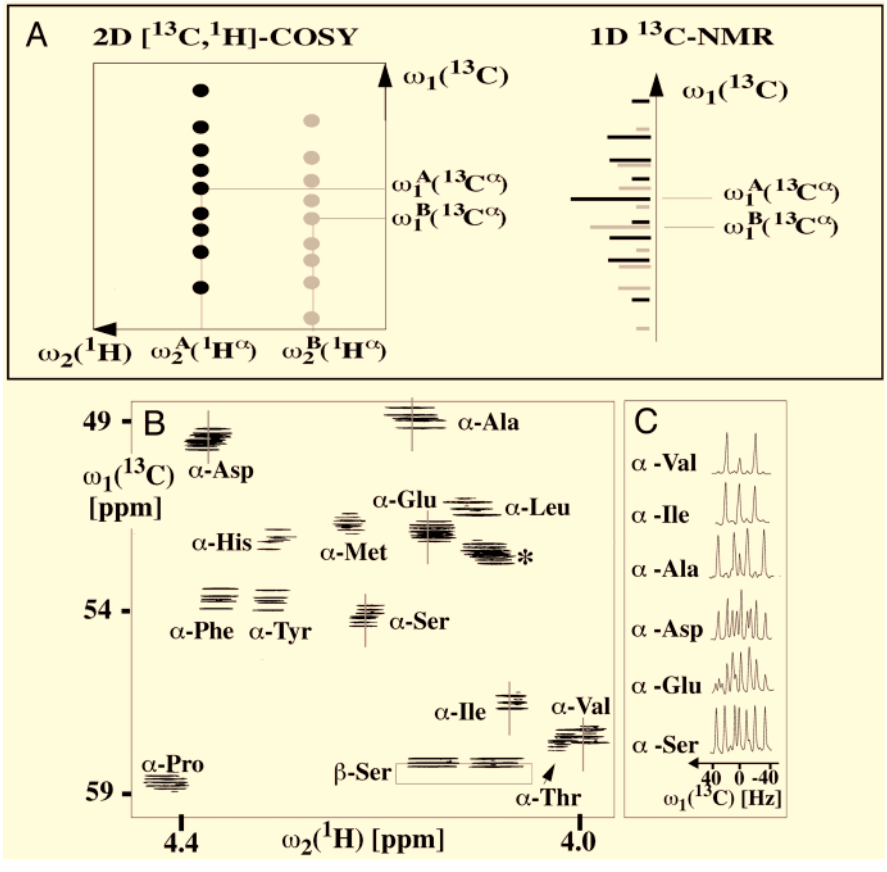

FIG. 2. (A) Illustration of increased spectral resolution in 2D NMR spectroscopy. The signals of two carbons that overlap in the 1D ^{13}C NMR spectrum (on the right) are resolved according to the chemical shift of their attached protons in the 2D [^{13}C,^{1}H]-HSQC spectrum (on the left). (B) Region of a 2D [^{13}C,^{1}H]-COSY spectrum containing the $^{13}C^{\alpha}$ $-^{1}H^{\alpha}$ cross peaks of all amino acids except glycine in a hydrolysate of cellular protein (Wüthrich *et al*., 1992; Szyperski *et al*., 1996). The resonance assignments are given using the three-letter code of the amino acids and greek letters for the carbon positions. (C) Cross sections taken along $\omega_1(^{13}C)$ at the broken vertical lines in (B), showing the ^{13}C–^{13}C scalar coupling fine structures of selected peaks. The asterisk indicates the overlapping cross peaks belonging to Lys-α and Arg-α.

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tion of the minimal medium (Fig. 1; see also Szyperski, 1998). This yields a uniform ^{13}C enrichment for all carbon positions. Hence, when ^{13}C NMR spectroscopy is used to analyze the labeling pattern, the ^{13}C–^{13}C spin–spin scalar coupling fine structure becomes the key observable that provides the relative abundance of ^{13}C-isotope isomers (isotopomers). These are directly linked to the extent to which a certain carbon atom is attached to carbons stemming from the same source molecule.

Efficient Analysis of ^{13}C–^{13}C *Scalar Coupling Fine Structures*: 2D [^{13}C,^{1}H] -*Correlation NMR Spectroscopy*

2D heteronuclear single-quantum coherence [^{13}C,^{1}H]-correlation NMR spectroscopy (2D [^{13}C,^{1}H]-COSY; Bodenhausen and Ruben, 1980) offers the most sensitive approach to assess the ^{13}C–^{13}C spin–spin scalar coupling fine structures: it is about five times more sensitive than standard 1D ^{13}C NMR spectroscopy performed with insensitive nuclei enhanced by polarization transfer (INEPT) (Ernst *et al*., 1987; for a recent review see Szyperski, 1998). Moreover, the two-dimensional dispersion of ^{13}C resonances enables one to analyze mixtures of metabolites without prior separation of the components: the ^{13}C resonances of the amino acid mixture would overlap in 1D ^{13}C NMR spectra (Fig. 2).

Beyond ^{13}C *Isotopomer Analysis*: *Tracing Carbon–Carbon Connectivities in a Bioreaction Network*

To elucidate the interconversion of source molecules that are fed into the bioreaction network into the metabolic precursors, the quantitative analysis of the ^{13}C fine structures must eventually unravel to which extent a certain carbon atom has neighboring carbons that originate from the same source molecule of glucose. This can be accomplished using a system of probabilistic equations (Szyperski, 1995) that allows calculation of the expected ^{13}C scalar coupling fine structure for a given "intact fragment" (being defined as a contiguous carbon fragment originating from a single source molecule). The calculated multiplet patterns take into account the background from the natural ^{13}C isotope abundance in the nonenriched source molecules, as well as the statistical recombination of [U-^{13}C] fragments due to finite dilution. Subsequently, the observed ^{13}C scalar coupling fine structure is decomposed according to the fine structures calculated for all possibly occurring intact fragments, thus yielding their relative abundance. Eventually, sums of such abundances represent the fraction of a given carbon–carbon connectivity that was cleaved due to the action of metabolism. This allows one to selectively trace such connectivities in the bioreaction network (Fig. 3).

Cellular Biomass: *A Readily Accessible Storage Device for* ^{13}C-*Labeled Metabolites*

An indirect approach to unravelling the labeling pattern of intermediary metabolites exploits the fact that their isotopomeric composition determines the labeling of the anabolic products generated by primary metabolism, *i.e*., amino acids, nucleosides, or secondary metabolites [for recent reviews see Szyperski (1998) and references therein]. Analysis of these products, in conjunction with the mapping of their carbon skeletons to those of the intermediates (see Fig. 6 in Szyperski, 1998), thus enables the determination of ^{13}C-labeling patterns of the intermediates. The richest source of information is the proteinogenic amino acids, which are linked to eight intermediates favorably spread over the network of central carbon metabolism (Stryer, 1995; Szyperski, 1995). Consequently, several major biosynthetic pathways can be analyzed in a single experiment. A major advantage of this indirect, biomass-oriented approach emerges when amino acids are derived from cellular protein, which represents about 55% of the biomass of an average *Escherichia coli* cell (Neidhardt *et al*., 1996) The protein essentially acts as a large storage device in which the labeling patterns of the intermediates accumulate during cellular growth, and direct acid hydrolysis of the biomass allows one to readily extract the labeling information. This indirect avenue to the investigation of glycolysis, pyruvate metabolism, the tricarboxylic acid cycle, and the pentose phosphate pathway, which is complemented by the analysis of C_1 metabolism via serine and glycine (Szyperski, 1995), results in a sensitivity enhancement, *i.e*., higher yields of molecules carrying the informative ^{13}C-labeling patterns, by three orders of magnitude or more (Szyperski, 1998). Finally, it is also of outstanding practical importance for NMR spectroscopic analyses that the proteinogenic amino acids exhibit sufficient ^{13}C chemical shift dispersion (Wüthrich, 1976). This ensures that strong ^{13}C–^{13}C scalar coupling effects do not have to be considered for data interpretation when using a modern high-field NMR spectrometer (Szyperski, 1995).

Two Targets of a Single Experiment: *Network Topology and Flux Ratios*

Once the observed fine structures have been translated into fragment abundances (Fig. 3), the breakdown of the carbon skeleton of the source molecule can be interpreted. This allows the identification of the metabolic pathways that are activated under the physiological conditions of the experiment (see "Selected Applications"), as well as the derivation of ratios of metabolic fluxes (Fig. 4). The data also allow the identification of irreversible reaction steps in the bioreaction network and semi-quantitative assessment

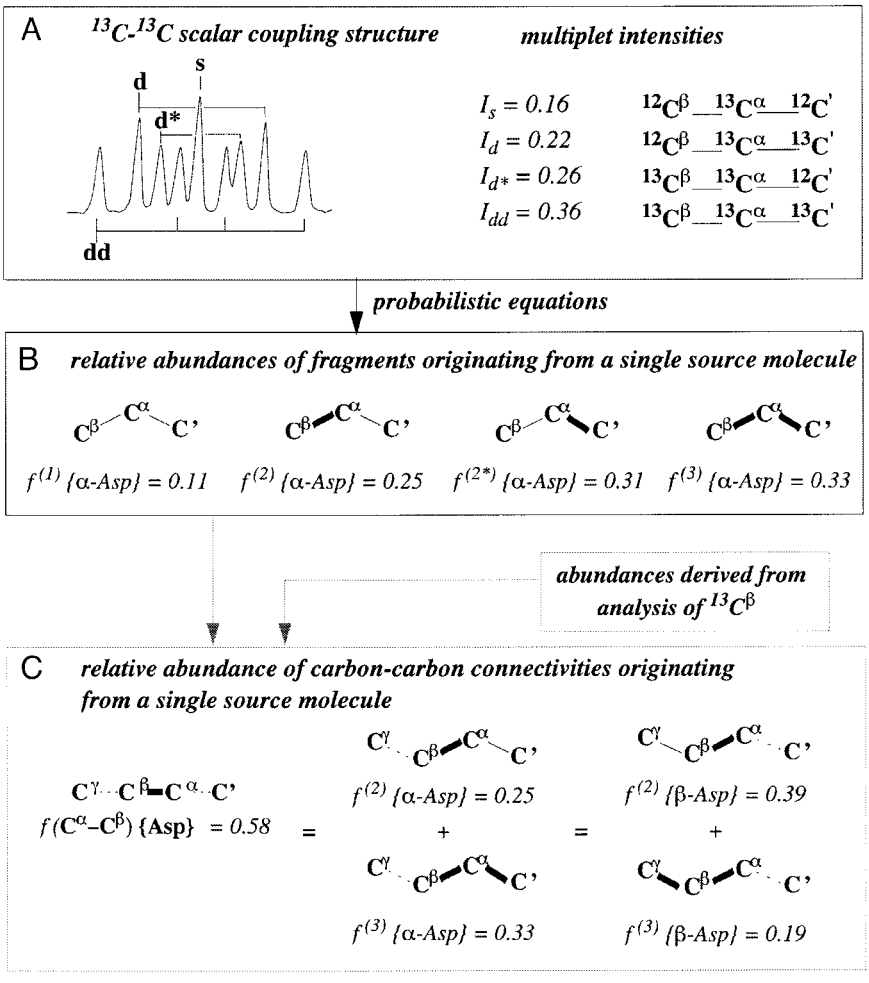

FIG. 3. Determination of relative abundances of carbon–carbon connectivities from the ^{13}C–^{13}C scalar coupling fine structure. (A) Integration of the fine structure yields the relative intensities of the multiplet components, and thus the relative abundance of the corresponding isotopomers (shown on right). I_s, I_d, I_{d*}, and I_{dd} indicate the relative intensities of the singlet, a doublet split by a small one-bond scalar coupling, a doublet* split by a large one-bond scalar coupling, and a corresponding doublet of doublets. As an example, the fine structure detected for the α-carbon of Asp is shown (see also Fig. 2). (B) The relative abundances of intact carbon fragments arising from a single source molecule of glucose are calculated using a system of probabilistic equations (Szyperski, 1995). Connectivities arising from a single source molecule are depicted in bold. In analogy to the notation for the multiplets, the values $f^{(1)}$, $f^{(2)}$, $f^{(2*)}$, and $f^{(3)}$ denote the fraction of Asp molecules that contain, respectively, an α-carbon without neighbor from the same source molecule, a C^{α}–C^{β} fragment, a C^{α}–C = O fragment, and a C^{β}–C^{α}–C = O fragment originating from a single source molecule, respectively. (C) When focusing on a certain carbon–carbon connectivity, *e.g.*, the C^{α}–C^{β} connectivity in Asp, sums of relative abundances are calculated to obtain the corresponding abundance $f(C^{\alpha}\text{–}C^{\beta})\{\text{Asp}\}$. Since the same value must be obtained when the β-carbon is analyzed (see text), we obtain that $f(C^{\alpha}\text{–}C^{\beta})\{\text{Asp}\} = (f^{(2)} + f^{(3)})\{\alpha - \text{Asp}\} = (f^{(2)} + f^{(3)})\{\beta - \text{Asp}\}$.

of exchange reactions (Szyperski, 1995; Szyperski *et al.*, 1996; Sauer *et al.*, 1997; Szyperski, 1998). Since the resulting flux ratios provide detailed insights into the *in vivo* flux distribution, we have proposed the term **meta**bolic **f**lux **r**atio (METAFoR) analysis (Sauer *et al.*, 1999).

Computer-Aided Data Analysis: The Program FCAL

Since 45 ^{13}C–^{13}C scalar coupling fine structures extracted from 2D [^{13}C,^{1}H]-COSY have to be evaluated for each BDF ^{13}C-labeled sample (for a visual impression, see Fig. 2), efficient routine application requires strong com-

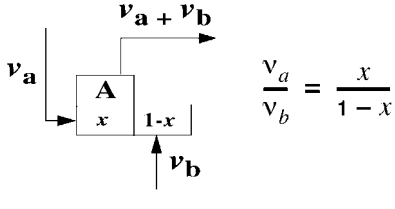

FIG. 4. Derivation of flux ratios from a BDF ^{13}C-labeling experiment. The NMR data provide the fractions of the total pool of molecules of metabolite **A**, x, and $1 - x$, originating either from pathway **a** or pathway **b**. Provided that these fluxes, as well as the efflux $v_a + v_b$, are irreversible, the flux ratios are identical to the ratios of the corresponding fractions.

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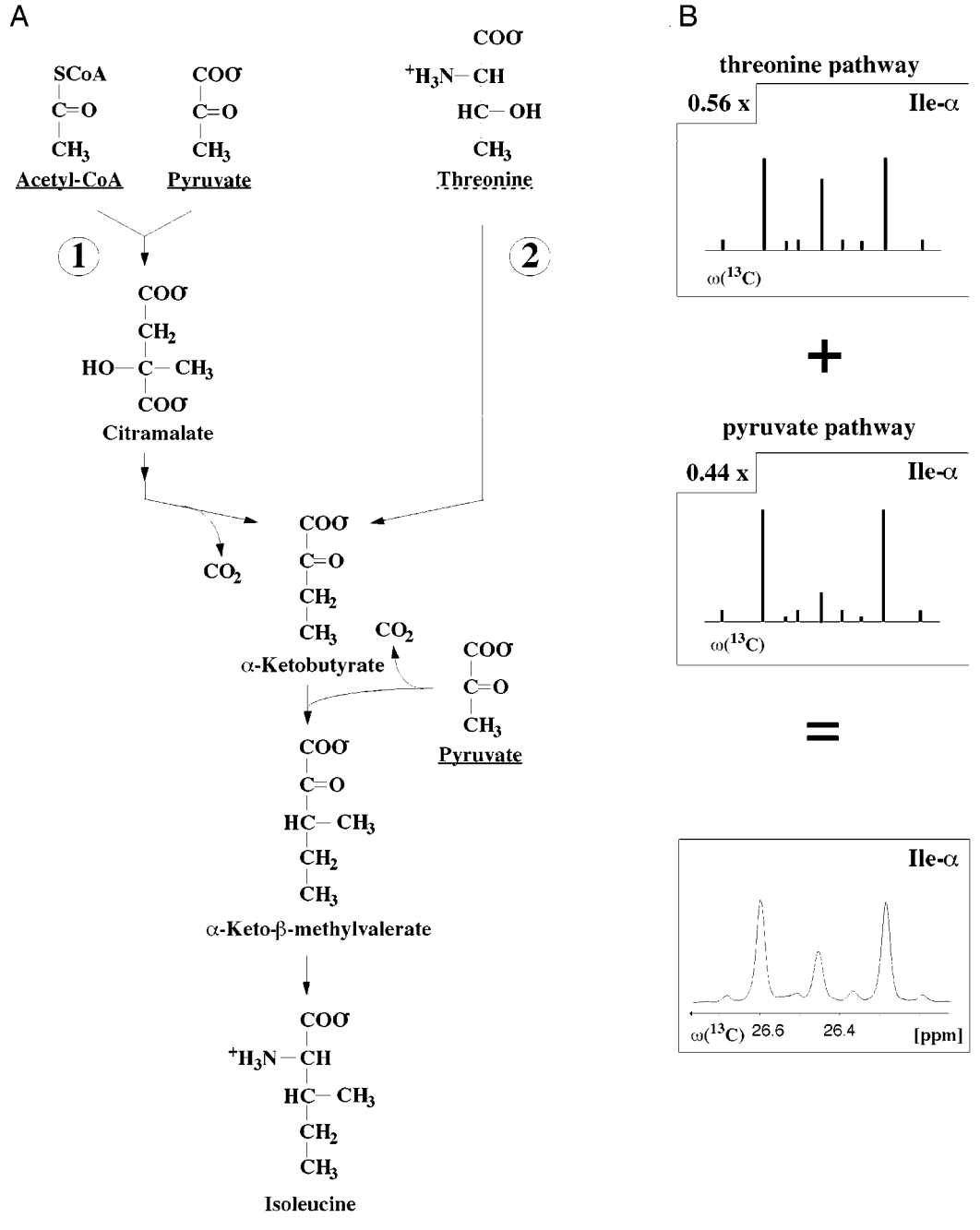

FIG. 6. (A) Isoleucine biosynthesis in *Haloarcula hispanica* (Hochuli *et al*., 1999). Pyruvate and acetyl-CoA serve for Ile synthesis *via* the so called "pyruvate pathway" (route 1) (Charon *et al*., 1974), while threonine and pyruvate are the precursors for Ile synthesis *via* the "threonine pathway" (route 2) (Umbarger, 1978). (B) Decomposition of the experimental ^{13}C–^{13}C scalar coupling fine structures detected for the α-carbon resonance of Ile into contributions from the threonine and pyruvate pathways, respectively. The stick diagrams represent the fine structures that would be expected if only a single pathway were operational, and the experimental cross section at the bottom was taken along $\omega_1(^{13}C)$ from a 2D [^{13}C,^{1}H]-COSY spectrum. Accordingly, 56 and 44% of isoleucine are synthesized *via* the threonine and pyruvate pathways, respectively.

puter support for the data analysis. To meet this objective, we have implemented the new program FCAL (**f**lux-ratios from **c**orrelated **a**mino acid ^{13}C-**l**abelling patterns). First, FCAL (Fig. 5) performs a nonlinear least-squares fit of the parameters describing a cross peak in 2D [^{13}C,^{1}H]-COSY (*i.e.*, ^{13}C and ^{1}H chemical shifts, ^{13}C–^{13}C scalar couplings, volumes and linewidths of the multiplet components, and ^{13}C isotope effects on the ^{13}C chemical shifts) to the experimental data. Suitable starting values for these parameters are retrieved from a library that has been generated from 2D [^{13}C,^{1}H]-COSY spectra recorded with a very high signal-to-noise ratio at the standardized NMR measurement conditions employed for routine analyses. Subsequently, the peak volumes are translated into relative abundances of intact fragments (Szyperski, 1998), which are checked for self-consistency. For example, the relative abundance of C^{α}–C^{β} connectivities must be equal when extracted from either the C^{α} or the C^{β} signal (Fig. 3). Moreover, the abundances must agree with the assumed bioreaction network. Finally, FCAL calculates metabolic flux ratios (Szyperski, 1995; Szyperski *et al.*, 1996; Sauer *et al.*, 1997; Szyperski, 1998; Sauer *et al.*, 1999), and the Gaussian law of error propagation is employed to estimate the experimental uncertainties.

SELECTED APPLICATIONS

Assessing the Bioreaction Network Topology: A Split Pathway for Amino Acid Biosynthesis in a Halophilic Archaeon

As indicated above, knowledge of the network of active biochemical pathways is a prerequisite to monitor *in vivo* fluxes. We identified the reactions catalyzed by the malic enzyme and phospho*enol*pyruvate carboxylase in *Bacillus subtilis*, which were previously considered to be inactive under the growth conditions chosen for the particular experiment (Sauer *et al.*, 1997). These findings pointed out that despite the large body of data available for "model microbes" such as *E. coli* (Neidhardt *et al.*, 1996) or *B. subtilis* (Sonenshein *et al.*, 1993), a direct experimental assessment of the network topology is often required to derive an appropriate metabolic model. This point has to be stressed further when approaching much less well-characterized organisms, *e.g.*, thermophilic or halophilic archaea living under extreme environmental conditions. Due to their unique metabolism (Davis, 1998), these species are of high potential interest for applications in biotechnology.

As an illustration, we sketch here selected results obtained when studying the amino acid biosynthesis in the halophilic archaeon *Haloarcula hispanica* (Hochuli *et al.*, 1999; Fig. 6. BDF ^{13}C labeling was achieved by growing the cells in a minimal medium containing a mixture of 90% unlabeled and 10% [U–^{13}C]-labeled glycerol as the sole carbon source in a batch culture, and the biomass was harvested at the end of the exponential growth phase. The experimental data revealed that most amino acids were synthesized according to pathways commonly found in eucarya and bacteria. In contrast, analysis of the ^{13}C–^{13}C scalar coupling fine structure detected for the α-carbon of Ile strongly suggested that the so-called "threonine" and "pyruvate" pathways for isoleucine biosynthesis operate in a split fashion (Fig. 6). This hypothesis has subsequently been confirmed by a ^{13}C-labeling experiment using uniformly ^{13}C-labeled threonine (Hochuli *et al.*, 1999). Intriguingly, neither the threonine pathway nor a split pathway for amino acid biosynthesis in general have previously been described for archaea. Moreover, the quantitative analysis also provides the flux ratio through the threonine and pyruvate pathways; *i.e.*, 56 and 44% of isoleucine was synthesized *via* the threonine and pyruvate pathways, respectively.

Assessing the in vivo *Flux Distribution: Metabolic Flux Ratio (METAFoR) Analysis*

We applied METAFoR analysis to several, partly metabolically engineered *E. coli* strains under various environmental conditions (Sauer *et al.*, 1999). This endeavor required the analysis of a large number of BDF ^{13}C-labeled samples. Figure 7 shows results obtained for wild-type *E. coli* cells grown either under carbon or nitrogen limitation (Fig. 7), which represent two largely different bioenergetic regimes. Most strikingly, METAFoR analysis revealed that an almost twofold-higher fraction of oxaloacetate molecules were derived from phospho*enol*pyruvate in the nitrogen-limited case. Hence, the anaplerotic carboxylase reaction (Stryer, 1995) appears to become the dominant pathway for oxaloacetate generation in *E. coli* cells under nitrogen deprivation. Since the flux ratios, or bounds thereof, that were derived for the pentose phosphate pathway are rather similar in the two regimes, the data suggest that the cells respond primarily by adapting the regulation of the tricarboxylic acid cycle.

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