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In vivo measurement of synthesis rate of multiple plasma proteins in humans

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Jaleel, Abdul, Vandana Nehra, Xuan-Mai T Persson, Yves Boirie, Maureen Bigelow, and K. Sreekumaran Nair. In vivo measurement of synthesis rate of multiple plasma proteins in humans. *Am J Physiol Endocrinol Metab* 291: E190–E197, 2006. First published January 31, 2006; doi:10.1152/ajpendo.00390.2005.—Advances in quantitative proteomics have facilitated the measurement of large-scale protein quantification, which represents net changes in protein synthesis and breakdown. However, measuring the rate of protein synthesis is the only way to determine the translational rate of gene transcripts. Here, we report a technique to measure the rate of incorporation of amino acids from ingested protein labeled with stable isotope into individual plasma proteins. This approach involves three steps: *1*) production of stable isotope-labeled milk whey protein, oral administration of this intrinsically labeled protein, and subsequent collection of blood samples; *2*) fractionation of the plasma and separation of the individual plasma proteins by a combination of anion exchange high-pressure liquid chromatography and gel electrophoresis; and *3*) identification of individual plasma proteins by tandem mass spectrometry and measurement of stable isotopic enrichment of these proteins by gas chromatography-mass spectrometry. This method allowed the measurement of the fractional synthesis rate (FSR) of 29 different plasma proteins by using the same precursor pool. We noted a 30-fold difference in FSR of different plasma proteins with a wide range of physiological functions. This approach offers a tremendous opportunity to study the regulation of plasma proteins in humans in many physiological and pathological states.

proteomics; plasma protein synthesis; phenylalanine; milk proteins; stable isotope

PLASMA PROTEINS play crucial physiological roles, and the alterations of certain plasma proteins indicate specific pathological states. Some of these changes are biomarkers of clinical disorders such as coronary artery disease (2) and cancer (25). However, only a limited number of plasma proteins have been measured and identified as markers of common clinical conditions, and the regulation of their plasma levels remains to be clearly defined. Recent technology based on in vitro stable isotope labeling methods (18) permits relative quantification of multiple plasma proteins. However, such techniques for measuring the relative concentrations of individual plasma proteins and peptide patterns in plasma do not allow us to understand the regulation at the molecular levels of plasma protein production and the maintenance of their levels. Gene array technology allows simultaneous measurement of the transcript levels of all known genes if appropriate tissue samples are available (26). This approach allows quantification of the transcript level changes of multiple genes and various functional clusters in response to physiological interventions and pathological states. There are, however, substantial discrepancies between changes in transcript levels and protein expression levels (9, 15–17, 19). The potential explanation for these discrepancies is the rapid turnover (synthesis and degradation) of proteins that may occur in many conditions so that concentrations of proteins may not reflect the actual synthesis rate, representing the translational rates of gene transcripts (28). It is therefore important not only to know the transcript and protein expression levels but also the rate at which the proteins are synthesized.

The measured abundance of a protein is the net result of its synthesis and breakdown. If these two processes are in equilibrium (despite upward or downward changes), the net concentration of the protein remains unchanged. During acute intervention, such as during a meal, although there is an increase in the rate of synthesis of specific proteins, such as albumin (11), there are no measurable changes in concentrations of many proteins because of their relatively slow turnover. Furthermore, protein quantification techniques are not sufficiently sensitive to detect changes that occur in very low abundance proteins, which may have a fast turnover rate. All of these limitations of in vitro labeling methods have led to the development of in vivo labeling of proteins for protein quantification, which has thus far only been applied to single-cell organisms (10, 29), cells in culture (10, 30), and to a limited extent in multicellular organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* (24). Proteomic strategies have also been developed for the relative measurement of protein turnover in chickens (12) and in cultures of single-cell organisms such as yeast (35) and bacteria (8). However, the aforementioned approaches require a high enrichment of proteins, which can be achieved by feeding animals for long periods with highly enriched isotopes or in single-cell organisms that have rapid turnover. Moreover, none of the above approaches provide a direct measurement of the synthesis rate of proteins, nor are they practical in human studies. Also, the aforementioned methods, as well the methods for protein quantification, do not have the sufficient precision for human studies, since these methodologies are highly dependent on the application of equal amounts of sample to be compared by mass spectrometry (MS) analysis. We have therefore developed an approach to simultaneously measure fractional synthesis rates of multiple plasma proteins and have demonstrated the method after a meal that contained intrinsically labeled proteins.

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EXPERIMENTAL PROCEDURES

Subjects. Six healthy adults (2 men and 4 women, average age 21.8 ± 0.7 yr) were enrolled in the study. Average body mass index was 22.8 ± 1.1 kg/m^2. Body composition was measured by dual-energy X-ray absorptiometry (23). Average fat-free mass (FFM) was 45.8 ± 4.4 kg. Only healthy people on physical examination and with normal fasting glucose and hepatic and renal function were studied.

Production of labeled milk protein. L-[*ring*-$^{13}C_6$]phenylalanine (Phe; 99 atom percent excess) was purchased from Cambridge Isotope Laboratories (Cambridge Isotope Laboratories, Andover, MA) and, after demonstrating a negative rabbit test, was made into sterile solution. The whey protein was intrinsically labeled by infusing L-[*ring*-$^{13}C_6$]Phe into lactating cows as previously reported (5). Briefly, a catheter was inserted percutaneously into the right jugular vein of the cow, and [*ring*-$^{13}C_6$]Phe was infused at a rate of 3 mg·kg^{-1}·h^{-1} using a peristaltic pump for 36 h. The cow was milked every 6 h for 36 h during the infusion and for another 24 h after discontinuation of the infusion. The milk collected during the period of isotope infusion and 24 h after the infusion was pooled. The whey protein was separated using a standardized ultrafiltration technique as previously described (5). The whey protein was then freeze-dried and stored for the experiments. The Phe concentration in whey protein was estimated as 4%, as was previously reported (37). Isotopic enrichment of [$^{13}C_6$]Phe whey milk protein was determined by gas chromatography-quadrupole mass spectrometry (GC-QMS). The whey protein was hydrolyzed overnight, at 110°C, using 6 M hydrochloric acid as previously described (3). The technique used for processing of amino acids and GC-QMS analysis was the same for plasma free Phe and the amino acid derived from hydrolyzed whey protein. The isotopic enrichment of whey protein was estimated to be 17.8 molar percent excess (MPE).

Experimental design. The study protocol was reviewed and approved by the Mayo Foundation Institutional Review Board, and informed consent was obtained from each participant prior to the study. FFM was used for estimating meal energy and protein content. For 3 days before the study, all subjects received a weight-maintaining diet consisting of 20% protein, 50% carbohydrate, and 30% fat, which was provided by the General Clinical Research Center (GCRC) at Mayo Clinic. All subjects received a standard meal by 8 PM on the day before the study and then fasted until the morning in the GCRC. Ingestion of water was allowed. The femoral artery, femoral vein, and hepatic vein were catheterized by a vascular radiologist. Samples from the femoral vein were used for a separate set of analyses. Catheter sheaths were placed in the right femoral artery and femoral vein through the arterial sheath. Hepatic vein catheters were inserted under fluoroscopic control, and the approximate position was confirmed by use of an injectable, nonionic contrast medium. A slow infusion of normal saline maintained the patency of the catheters. The femoral artery and hepatic lines were used for collecting blood samples. After baseline blood samples were collected, subjects ingested 0.626 g/kg FFM of whey protein intrinsically labeled with [*ring*-$^{13}C_6$]Phe in combination with a similar amount of casein and 1.8 g/kg FFM of lactose. This provided a total of 1.25 g protein/kg FFM. Blood samples from the femoral artery and hepatic vein were collected at baseline and then every 20 min for 420 min. Baseline and 400-min samples from the femoral artery were used to calculate enrichment for individual plasma proteins. The study was originally designed as a part of a larger physiological experiment, and the additional results will be reported separately.

Separation of individual plasma protein. Femoral arterial plasma samples from baseline and at 400 min of all subjects were used for the analysis of isotopic enrichment of individual plasma proteins. Each plasma sample was subjected to immunoglobulin removal (Affigel Protein-A; Bio-Rad, Hercules, CA) followed by albumin removal (SwellGel Blue; Pierce, Rockford, IL) to reduce the content of these highly abundant proteins. The recovery of proteins following depletion of plasma samples was ~20%, based on the estimated total protein concentration in plasma before and after depletion.

Each depleted plasma sample was fractionated further by use of anion exchange HPLC. Samples were loaded onto a PolyWAX LP (PolyLC Columbia USA, Columbia, MD) anion exchange column (9.4 mm × 10 cm) and eluted using a Waters 600E HPLC system. The column was washed for 5 min with *buffer A* (20 mM Tris, pH 7.5) followed by a gradient of 0 to 100% *buffer B* (0.8 M sodium acetate in *buffer A*) over 120 min. The flow rate was 2 ml/min, and the optical density (OD) was monitored at 280 nm. Fifty fractions of 5 ml each were collected. Each fraction was precipitated separately using 10% trichloroacetic acid. The precipitate from each fraction was dissolved in SDS sample buffer, and SDS-PAGE was performed on an 8–15% acrylamide gel of 1.5 mm thickness. Each time point had 50 HPLC fractions, and a total of 600 fractions from six subjects were obtained and were subjected to gel electrophoresis (50 fractions were electrophoresed at one time with 25 fractions each on a single gel). The gels were later stained with Coomassie blue. Pairs of 37 gel bands, which were the best resolved and intense from both time points, obtained from 50 fractions of each time point were excised and analyzed for protein identification by tandem mass spectrometry (LC-MS-MS) and for isotopic enrichment by GC-QMS. Figure 1 displays only the lines (HPLC fractions) showing the gel bands that were used for the study. The rest of the HPLC fractions either did not produce any protein gel bands or showed gel bands that were not considered optimal for analysis because of the difficulty in reaching correct protein identification. Although we analyzed a total of 37 gel bands from each sample (50 HPLC fractions), only 29 were identified as single protein and therefore used for isotopic measurement.

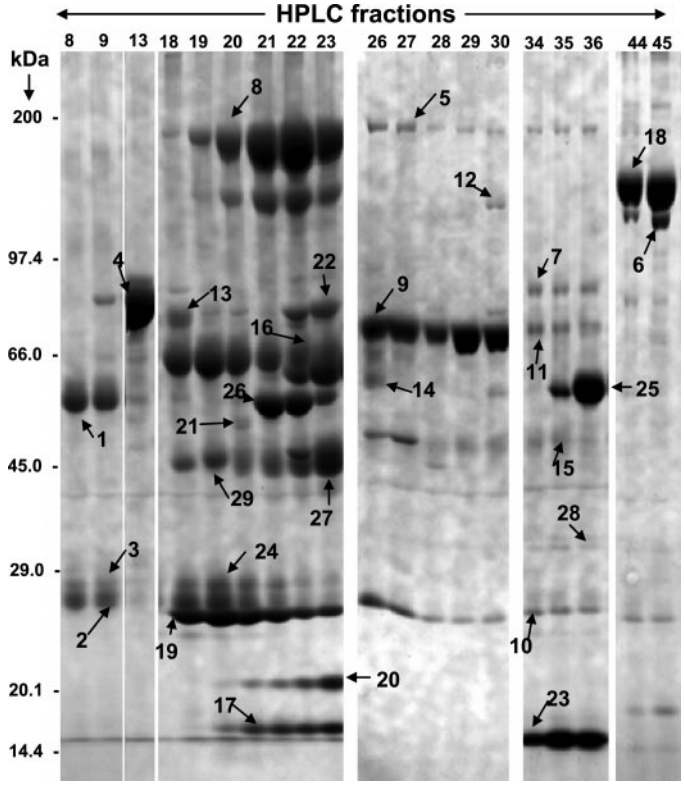

Fig. 1. SDS-PAGE of anion exchange HPLC fractions of plasma proteins. Plasma proteins were first fractionated into 50 HPLC fractions, and proteins from each fraction were precipitated and subjected to SDS-PAGE on 8–15% gradient gels using the Bio-Rad Protean-II system. Gels were stained with Coomassie blue to visualize protein gel bands. The figure displays only the lines (HPLC fractions) showing the gel bands that were used for protein identification and isotopic enrichment analysis. The gel bands are numbered to match the protein serial numbers in Tables 2 and 3.

Protein identification by LC-MS-MS. Gel bands were subjected to in-gel reduction and alkylation, followed by trypsin digestion, as previously described (21, 36). Peptides were extracted three times, first by adding 2% trifluoroacetic acid (TFA) in water to the digest mixture and then by an equal volume of 60% acetonitrile-0.1% TFA-40% water followed by a final extraction with 100% acetonitrile. Extracts were dried by evaporation (Savant Speedvac) to remove the organic solvent. Samples were dissolved in 0.1% formic acid-2% acetonitrile solution just before injection into the mass spectrometer.

MS analyses were done on a linear ion-trap quadrupole mass spectrometer (LTQ; Thermo Electron, Franklin, MA). The digested peptides were introduced into the LTQ through an automated nanoscale liquid chromatography system (LC Packings, Sunnyvale, CA). The chromatographic separation was performed on a 100-μm inside diameter × 15-cm C_{18} column (Zorbax; Agilent Technologies, Palo Alto, CA) with a linear gradient elution from 100% *buffer A* (0.1% formic acid-acetonitrile, 98:2, vol/vol) to 60% *buffer B* (0.1% formic acid-acetonitrile, 2:98, vol/vol) in 60 min. The collected data were compared against a nonredundant human database by use of TurboSequest software (University of Washington). Peptide mass tolerance and fragment mass tolerance were set at 1.4 and 0.5 Da, respectively. Identification of individual proteins was confirmed by triplicate analysis of gels bands of baseline samples from three different study subjects.

The tissue origin of each identified proteins as well as their functions were obtained from Human Protein Reference Database (33) and Swiss-Protein database.

Isotopic enrichment of individual plasma proteins derived from gel bands. Isotopic enrichment of Phe derived from hydrolysis of plasma proteins by GC-QMS was measured as described previously (7, 32). For GC-QMS analysis, gel pieces were washed several times with deionized water and hydrolyzed overnight at 110°C in 6 M hydrochloric acid. Amino acids from the hydrolyzed gel pieces were purified over an AG-50W cation exchange column, eluted with 4 M ammonium hydroxide, and dried by evaporation. Methyl esters were made before acylation with heptafluorobutyric anhydride. Derivatives were dried and resuspended in ethyl acetate for GC-QMS analysis on a Triple Stage Quadrupole (Finnigan TSQ 7000; Thermo Electron) under positive chemical ionization with ammonia gas. Amino acid (Phe ester) separation was carried out on a DB5-ms 30 m × 0.25 mm × 0.25 μm capillary column (Agilent Technologies) in the GC oven with helium flow rate of 3 ml/min and the temperature ramp as follows: 100–152°C at 5°/min, 152–154°C at 1°/min, and 154–300°C at 20°/min. Selected ion monitoring was performed in profile mode at *m/z* 396 ($M + 3$) and *m/z* 399 ($M + 6$) for [^{12}C]Phe and [$^{13}C_6$]Phe, respectively (7, 32). Enrichment (molar percent excess, MPE) was calculated using an equation obtained from a six-point calibration curve. The regression analysis of theoretical and measured enrichment gave regression coefficient (r^2) of 0.9998. Replicate measurements of isotopic enrichment of proteins collected from five separate gel bands after gel electrophoresis (as described above) were performed to determine reproducibility (Table 1). Ten replicate measurements in each of five different protein bands showed a coefficient of variation ranging from 3.4 to 6.5%.

Isotopic enrichment of free Phe in plasma. Isotopic enrichment of free [$^{13}C_6$]Phe in plasma was determined by GC-QMS. We analyzed 23 samples (at −20 min and every 20 min until 420 min, i.e., 23 samples from each subject and total 276 samples). Each plasma sample (0.1 ml) was acidified using 0.1 ml of 50% acetic acid, and this mixture was passed through an AG-50W cation exchange column. After the column was washed with water, the amino acids were eluted with 4 M ammonium hydroxide followed by evaporation to dryness using a centrifugal evaporator. The *tert*-butyldimethylsiloxy derivative was prepared and, after removal of excess solvent, resuspended in n-decane for analysis by GC-MS (5973N GC/MS; Agilent Technologies) under electron ionization conditions. Chromatographic separation was carried out on a 30 m × 0.25 mm × 0.25 μm DB5-ms capillary column (Agilent Technologies) using helium as carrier gas at 2 ml/min. A ramped temperature program was used: 120–175°C at 20°/min, 175–195°C at 10°/min, and 195–300°C at 30°/min. A limited scan of the $[M - 57]^+$ region was performed, *m/z* 336–344 with 10 ms dwell for each ion and triplicate injections of each label for each plasma sample compared with similar profiles collected for natural abundance Phe. The MPE of [$^{13}C_6$]Phe was calculated above background, as previously described (20).

Table 1. *Reproducibility of very low level isotopic enrichment measurements from gel bands*

Band	L-[*ring*-$^{13}C_6$]Phe, MPE*	CV, %
1	0.0640±0.0008	3.73
2	0.0523±0.0008	5.10
3	0.1131±0.0030	5.75
4	0.2901±0.0053	3.41
5	0.1246±0.0025	6.46
		4.89±0.58 (mean±SE)

*Values are means ± SE; $n = 10$ replicates. MPE, mole percent excess. One representative plasma sample at 400 min after meal ingestion was run 50 μg each in 10 wells on an 8–15% SDS-PAGE. Five separate gel bands (*1–5*) were excised from each of the 10 lanes of the gel, processed, and performed isotopic enrichment analysis on by GC-MS as described in EXPERIMENTAL PROCEDURES.

Calculation of FSR of plasma proteins. We subtracted the baseline natural isotopic abundance of [$^{13}C_6$]Phe in plasma proteins from those samples collected 400 min after the meal (I_e). The integrated values for plasma [$^{13}C_6$]Phe isotopic enrichment of all time points after the administration of the labeled meal were used as the precursor enrichment (P_e). The calculation of protein FSR has been described previously (27). The equation used is as follows: FSR (%/h) = $[I_e/(P_e \cdot t)] \cdot 100$, in which t represents time (6.67 h).

We used isotopic enrichment (integrated values for 6.67 h) of hepatic venous plasma (P_e) as precursor for FSR calculation of proteins derived from liver and femoral arterial plasma enrichment (Pe) to calculate FSR of proteins from tissues other than liver.

Statistical analysis. All values are expressed as means ± SE. Comparison of plasma Phe isotopic enrichment values between hepatic vein and femoral artery was analyzed using a paired *t*-test.

RESULTS

Isotopic enrichment of plasma Phe and Phe concentration. As expected, immediately following the ingestion of the meal, both arterial and hepatic venous isotopic Phe enrichment values and Phe concentration increased, and enrichment value remained above natural abundance until the end of the study (Fig. 2). In contrast, plasma Phe concentration returned near baseline by 360 min. Although both arterial and venous Phe concentrations were similar, the enrichment values were higher in the hepatic vein from 20 to 280 min (4.44 ± 0.24 average MPE for hepatic vein vs. 3.57 ± 0.17 for femoral artery, $P < 0.001$).

Isotopic enrichment of individual plasma proteins and precursors. The integrated values of femoral arterial [$^{13}C_6$]Phe enrichment were significantly lower (2.92 ± 0.28) than those of hepatic venous enrichment (3.57 ± 0.33, $P < 0.001$). The isotopic enrichment of each of the 29 proteins (Table 2) showed a wide range in enrichment values (0.03 to 0.66 MPE).

Identified proteins and their functions. The proteins identified with corresponding sequest score, percent coverage, and numbers of peptides are shown in Table 2. The functions, as well as the origins, of the proteins whose synthesis rates are measured are given in Table 3. The majority of the proteins

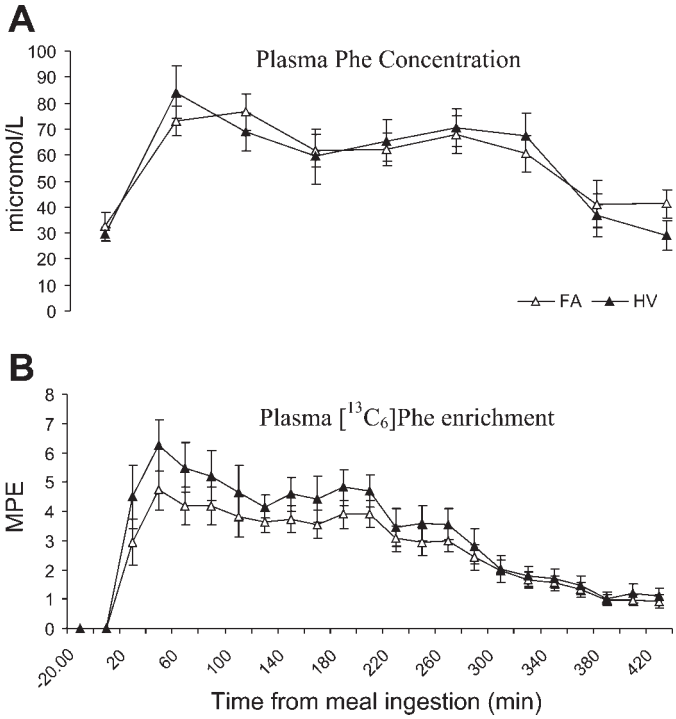

Fig. 2. Plasma Phe concentration and Phe isotopic enrichment following the ingestion of L-[*ring*-$^{13}C_6$]Phe-labeled whey protein. *A*: plasma Phe concentration from femoral artery (FA) and hepatic vein (HV) of 9 time points from 0 to 7 h. *B*: plasma [$^{13}C_6$]Phe enrichment from FA and HV starting from −20 min and followed by meal (0 min) for every 20 min up to 7 h. MPE, mole percent excess. Data points are means ± SE; $n = 6$.

identified are of liver origin, and others are derived from either B lymphocytes or the intestine.

FSR of plasma proteins. FSR (%/h) of 29 plasma proteins are given in Fig. 3 (1.13 ± 0.16 to 30.91 ± 6.75%/h), demonstrating an ~30-fold difference.

DISCUSSION

We measured in vivo FSRs of 29 plasma proteins in healthy people from the rate of incorporation of labeled Phe derived from intrinsically labeled milk protein administered orally. With this technique we were able to observe a 30-fold range in the synthesis rate of plasma proteins. The measurement of FSRs of proteins represents the best measurement of translational rates of gene transcripts, thus offering a potential opportunity to understand the in vivo regulation of specific genes. Unlike previous approaches, limited to the measurement of synthesis rates of only a few plasma proteins (13, 22), here we utilized a large-scale protein purification approach in combination with mass spectrometry to identify the purified proteins as well as to measure the stable isotopic abundance of Phe in these proteins. We measured postprandial synthesis rates of these proteins not only because the postprandial period is longer than the fasted state but also because the period represents the net anabolic phase.

The use of intrinsically labeled milk whey protein has some advantages in measuring postprandial FSR of plasma proteins. Both tracer amino acid and amino acids derived from the ingested protein appear simultaneously when ingested protein is labeled with the tracer. In contrast, free amino acid tracer

Table 2. *Plasma proteins identified and their respective [$^{13}C_6$]Phe enrichment*

Protein Gel Band	Protein or Plasma Sample	MPE	Sequest Score	Accession no.	No. of Peptides	%Coverage
1	Immunoglobulin heavy-chain constant region	0.03±0.00	69.16	10334541	7	28
2	Immunoglobulin kappa light chain	0.03±0.00	89.27	21669337	9	17
3	L chain L, crystal structure of tissue factor in complex with humanized Fab D3	0.05±0.01	198.29	18655500	20	26
4	TFHUP transferrin precursor	0.08±0.01	160.30	4557871	29	55
5	MAHU alpha2-macroglobulin precursor	0.05±0.01	520.29	4557225	52	68
6	Ceruloplasmin	0.06±0.01	280.23	4557485	28	17
7	MHHUM Ig mu chain c region, membrane-bound splice form, human	0.07±0.01	447.26	7428606	45	22
8	Alpha2 macroglobulin precursor	0.06±0.00	240.27	4557225	24	54
9	HSA in a complex with myristic acid and triiodobenzoic acid	0.07±0.00	160.25	4389275	16	15
10	Immunoglobulin J chain	0.08±0.01	40.16	21489959	4	34
11	A chain A, crystal structure of human serum albumin	0.11±0.01	309.26	3212456	31	26
12	Complement component 3 precursor; acylation-stimulating protein cleavage	0.13±0.02	70.20	4557385	7	28
13	Hemopexin	0.11±0.02	60.21	386789	6	25
14	A1AT_human alpha1-antitrypsin precursor	0.14±0.03	290.28	1703025	29	28
15	Apolipoprotein A-IV precursor	0.14±0.02	200.22	4502151	20	48
16	Albumin precursor; pro0883 protein	0.15±0.05	189.24	23307793	19	70
17	Apolipoprotein A-II	0.13±0.03	100.19	4502149	10	17
18	KUHU ferroxidase (EC 1.16.3.1) precursor	0.17±0.01	279.23	1070458	28	26
19	Haptoglobin-related protein; haptoglobin-related locus	0.14±0.08	160.20	3337391	16	17
20	Apolipoprotein D, apoD	0.30±0.07	59.20	619383	6	34
21	NBHUA2 leucine-rich alpha-2-glycoprotein, human	0.27±0.08	330.20	72059	33	17
22	Alpha1B-glycoprotein	0.32±0.02	140.20	23503038	14	12
23	Transthyretin (prealbumin, amyloidosis type i)	0.24±0.06	240.30	4507725	24	54
24	Apolipoprotein A-1 precursor	0.17±0.02	439.27	4557321	44	64
25	Alpha2HS-glycoprotein	0.35±0.02	100.30	4502005	10	17
26	VYHUD vitamin D-binding protein precursor	0.38±0.04	159.20	139641	16	51
27	Haptoglobin	0.45±0.03	322.30	4826762	35	49
28	B chain B, the structure of human retinol-binding protein with its carrier protein	0.52±0.01	39.23	4558176	4	22
29	Zinc-alpha2-glycoprotein precursor	0.66±0.11	350.30	72059	35	34

Plasma proteins were identified by tandem mass spectrometry. Identification was confirmed by triplicate analysis of baseline samples from 3 different individuals. Sequest score, accession no., no. of peptides, and %coverage are from a representative analysis. Isotopic enrichment values (MPE ± SE) are from all 6 study subjects analyzed by GC-MS.

Table 3. *Plasma proteins, whose FSRs were calculated in the current study*

No.	Protein ID	Function	Tissue Specificity
1	Immunoglobulin heavy-chain constant region	Antigen binding and effector functions	B-lymphocytes
2	Immunoglobulin kappa light chain	Antigen binding and effector functions	B-lymphocytes
3	L chain L, crystal structure of tissue factor in complex with humanized Fab D3		
4	TFHUP transferrin precursor	Iron binding	Liver
5	MAHU alpha2-macroglobulin precursor	Protease inhibitor and cytokine transporter	Liver
6	Ceruloplasmin	Ferroxidase activity, amine oxidase activity, copper transport, and homeostasis	Liver
7	MHHUM Ig mu chain C region, membrane-bound splice form		B-lymphocytes
8	Alpha2 macroglobulin precursor	Protease inhibitor and cytokine transporter	Liver
9	Human serum albumin in a complex with myristic acid and tri-iodobenzoic acid		
10	Immunoglobulin J chain (*Homo sapiens*)	Assembly of polymeric immunoglobulins and transport of these across epithelial cell layers	Plasma cells
11	A chain A, crystal structure of human serum albumin		
12	Complement component 3 precursor;	Central role in the activation of the complement system	Liver hepatocytes
13	Hemopexin	Transportation of heme in the plasma and an important antioxidant	Liver
14	A1AT_HUMAN Alpha-1-antitrypsin precursor (Alpha-1 protease inhibitor)	Inhibitor of serine proteases.	Liver
15	Apolipoprotein A-IV precursor	Role in chylomicrons and VLDL secretion and catabolism	Small intestine
16	Albumin precursor	Maintenance of colloid osmotic pressure, binding, and transport free radical scavenging	Liver
17	Apolipoprotein A-II	Inhibits high-density lipoprotein remodeling and lipid-poor apolipoprotein A-1 formation	Liver
18	KUHU ferroxidase (EC 1.16.3.1) precursor	Ferroxidase activity, amine oxidase activity, copper transport, and homeostasis	Liver
19	Haptoglobin-related protein; Haptoglobin-related locus	Lytic activity	Liver
20	Apolipoprotein D, apoD	Transporter of small, hydrophobic ligands	Liver & intestine
21	NBHUA2 leucine-rich alpha-2-glycoprotein - human	Unknown function	Neutrophils
22	Alpha 1B-glycoprotein	Unknown function	Liver?
23	Transthyretin	Transports thyroxine and retinol-binding protein	Choroid plexus and liver
24	Apolipoprotein A-I precursor	Promotes cellular cholesterol efflux, binds lipids, and activates cholesterol acyltransferase	Liver and intestine
25	Alpha2-HS-glycoprotein; alpha-2HS-glycoprotein	Involved in endocytosis, brain development, and formation of bone tissue	Liver
26	VYHUD vitamin D-binding protein precursor	Binds vitamin D and its metabolites and binds G-actin to prevent its polymerization during cell injury	Liver
27	Haptoglobin	Binds free hemoglobin, is an acute-phase reactant, and also has immune modulatory function	Liver
28	B chain B, the structure of human retinol-binding protein	Carrier for retinol (vitamin A alcohol) in the blood	Liver
29	Zinc-alpha2-glycoprotein precursor	Stimulates lipid degradation in adipocytes	Liver & glandular epithelial cells

Corresponding functions along with their site of synthesis.

mixed with orally ingested protein appears and disappears faster than amino acids derived from ingested protein because of the time needed to digest the ingested protein by pancreatic enzymes (6). As a result, the use of free amino acid tracer in blood is a better representation of the actual precursor of protein synthesis in liver when the ingested protein is labeled with amino acid tracer. Another approach is using an intravenous free amino acid tracer to measure postprandial plasma protein synthesis. The problem with this approach is that a meal can dramatically dilute the intravenously administered tracer to low levels due to an increase in unlabeled amino acids appearing from meal proteins (14). The dilution of the tracer enrichment occurring in the hepatic circulation by the meal-derived amino acids transiting through the portal circulation cannot be fully assessed when the tracer is infused systemically. We circumvented this problem by having subjects ingest the tracer-bound protein [*ring*-$^{13}C_6$]Phe-whey as a meal.

We used the hepatic vein free Phe label as precursor for calculating FSR of liver-derived proteins, whereas for other proteins we used arterial label as the precursor. Although the Phe enrichment in hepatic vein and femoral artery was not in a steady state following meal ingestion, the average value is likely to represent the true precursor enrichment values. We used the same precursor values for FSR calculations of all plasma proteins, and therefore the fold differences that we reported are valid. We also determined the differences in isotopic enrichment between arterial and hepatic vein [$^{13}C_6$]Phe and found an average 18% lower enrichment in arterial plasma. This can result in some underestimation of FSR of liver-derived plasma proteins when arterial plasma Phe label is used as precursor. The lower isotopic enrichment of arterial Phe can be explained by unlabeled Phe appearing from degradation of proteins from the peripheral tissues. Theoretically, the hepatic vein that drains blood from liver is likely to

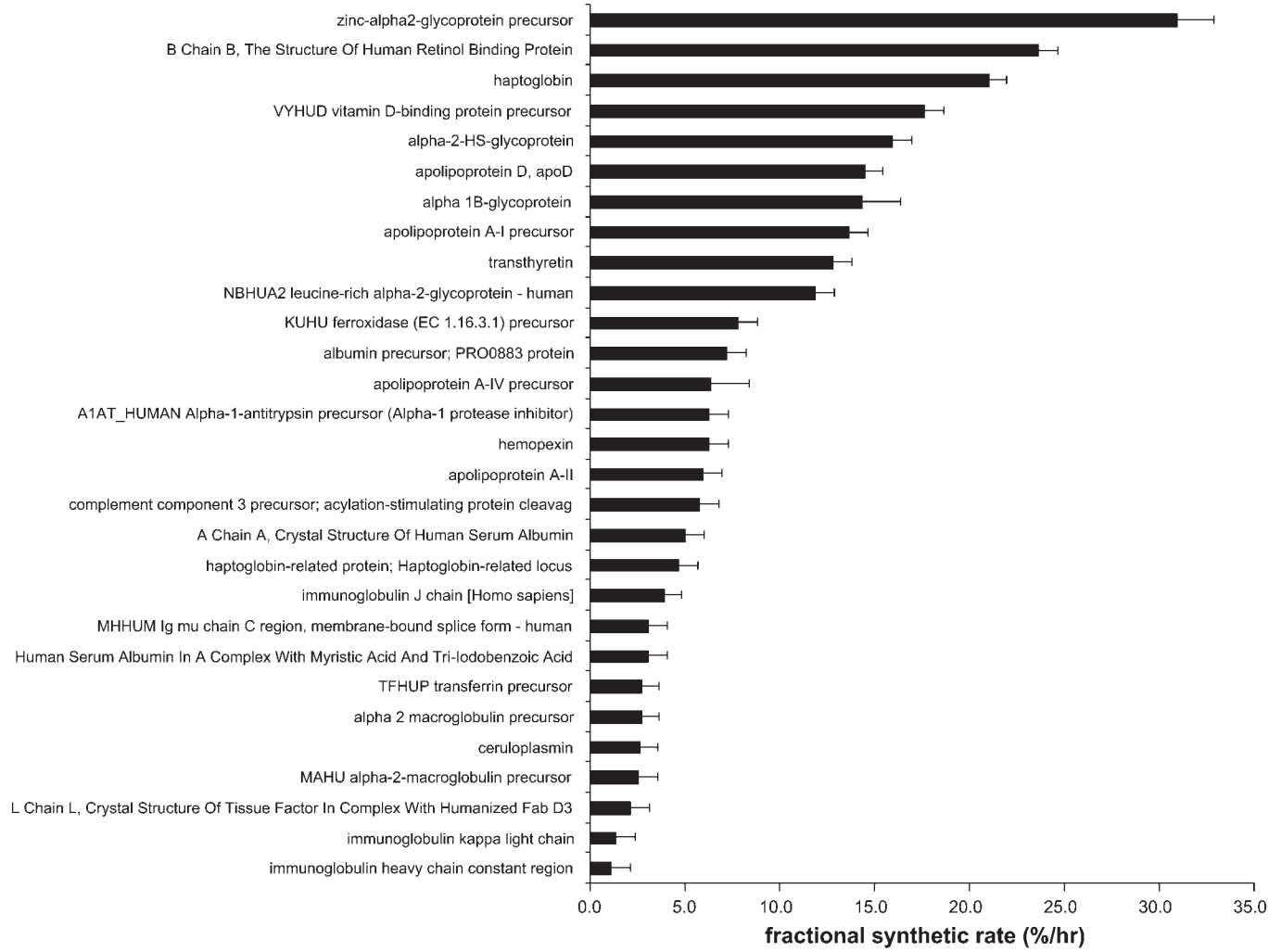

Fig. 3. Fractional synthesis rates (FSR) of 29 plasma proteins. Proteins are arranged in hierarchical order of their FSR, showing the highest value for zinc α2 glycoprotein and with immunoglobulin heavy-chain constant region showing the lowest. The FSR of each protein was calculated using precursor pool enrichment from either hepatic vein for proteins of hepatic origin or femoral artery for proteins of nonhepatic origin.

represent hepatic [$^{13}C_6$]Phe enrichment in the intracellular compartment of the liver. This assumption is supported by results from previous studies (1, 4) demonstrating that liver tissue fluid and tRNA leucine enrichment are best represented by arterial ketoisocaproate enrichment, which is not different from the hepatic venous leucine enrichment. We appreciate that it is not practical to measure hepatic venous plasma isotopic enrichment for most human studies. The current results demonstrated that the difference in enrichment of hepatic and peripheral Phe enrichment is small. It remains to be determined whether a similar relationship exists for other amino acid tracers as well.

In the present study, we chose anion exchange HPLC as the first dimension separation based on charge and SDS-PAGE as the second dimension to separate plasma proteins instead of a two-dimensional gel electrophoresis to obtain sufficient protein concentration in a single gel band for detection with GC-QMS. The individual plasma proteins were identified by MS-MS as described in EXPERIMENTAL PROCEDURES. In the present study, we selected only 29 gel bands, which were intense and resolved clearly, for analysis. With those bands, we could analyze ~10% of the known plasma proteins, which could be separated and identified using gel separation techniques (34). Our separation approach yielded individual proteins for the analysis. The combination of two one-step procedures used in the present study is not sufficient to isolate the maximal number of plasma proteins. The main difficulty to overcome is the complexity and tremendous variation in individual plasma protein abundance; e.g., albumin is 10^9-fold more abundant in plasma than troponin T. A combination of different ranges of liquid chromatography columns along with electrophoresis methods can separate and isolate medium- and low-abundance plasma proteins. However, the present report is the first, to our knowledge, to report in vivo synthesis rates of a large number of plasma proteins in humans. Ten replicate experiments from five separate gel bands demonstrated the high precision of the measurement, which offers the opportunity to detect small changes in plasma protein enrichment (Table 1).

We determined the function and origin of these plasma proteins (Table 3) from the Human Protein Reference Database (http://www.hprd.org/query) and Swiss-Protein database (http://www.expasy.ch/cgi-bin/sprot-search-ful). We found

that proteins with immunologic functions, such as immunoglobulin heavy-chain constant, and immunoglobulin κ-chain have more than 10-fold lower synthesis rates than some of the binding proteins, such as retinol-binding proteins and vitamin D-binding proteins. These binding proteins are critical in transporting nutrient molecules, such as vitamin D and retinol, or hormones, such as thyroxine. The production of these proteins is critical to preventing sudden increases in the concentration of free molecules with major functional consequences. In general, it appears that most of the proteins that we identified with a fast FSR are proteins, such as apolipoproteins, that are necessary for transferring lipids or fatty acids to different locations, or acute phase reaction proteins, such as haptoglobin.

The current approach offers the opportunity to determine the effect of various physiological factors, such as growth, aging, hormones, and substrates, as well as pathological states on the rate of synthesis of various circulating proteins. Because these changes are measures of the translational rates of transcripts to proteins, they can be used to obtain precise mechanistic information of the effect of physiological interventions and pathological states. It is conceivable that lack of changes in concentrations of proteins between two study conditions may occur if synthesis and degradation rates change in the same direction at the same magnitude. For example, the synthesis and degradation rates of a single protein or cluster of proteins with similar functions could increase 10-fold in a specific condition with a major effect on functions but no measurable changes in the protein concentrations.

In conclusion, the approach described in the present study enables the measurement of synthesis rates of multiple plasma proteins. Our study uses the labeling of ingested protein to measure the rate at which labeled amino acid is incorporated into multiple plasma proteins, thus enabling us to measure their synthesis rates following a meal. Similar techniques could be used to measure synthesis rates of multiple proteins in the postabsorptive state with intravenous administration of tracers, as has been reported by many authors for the measurement of one or two plasma proteins (31). In summary, we measured the postprandial synthesis rates of 29 plasma proteins by in vivo labeling of these proteins, thus demonstrating a 30-fold difference in their synthesis rates. Simultaneous measurement of transcript levels and FSR in the same tissue during interventions will allow one to determine whether the intervention affected translation or transcription.

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REFERENCES

1. **Ahlman B, Charlton M, Fu A, Berg C, O'Brien P, and Nair KS.** Insulin's effect on synthesis rates of liver proteins. A swine model comparing various precursors of protein synthesis. *Diabetes* 50: 947–954, 2001.
2. **Anderson L.** Candidate-based proteomics in the search for biomarkers of cardiovascular disease. *J Physiol* 563: 23–60, 2005.
3. **Balagopal P, Ford GC, Ebenstein DB, Nadeau DA, and Nair KS.** Mass spectrometric methods for determination of [13C]leucine enrichment in human muscle protein. *Anal Biochem* 239: 77–85, 1996.
4. **Barazzoni R, Meek SE, Ekberg K, Wahren J, and Nair KS.** Arterial KIC as marker of liver and muscle intracellular leucine pools in healthy and type 1 diabetic humans. *Am J Physiol Endocrinol Metab* 277: E238–E244, 1999.
5. **Boirie Y, Fauquant J, Rulquin H, Maubois JL, and Beaufrere B.** Production of large amounts of [13C]leucine-enriched milk proteins by lactating cows. *J Nutr* 125: 92–98, 1995.
6. **Boirie Y, Gachon P, Corny S, Fauquant J, Maubois JL, and Beaufrère B.** Acute postprandial changes in leucine metabolism as assessed with an intrinsically labeled milk protein. *Am J Physiol Endocrinol Metab* 271: E1083–E1091, 1996.
7. **Calder AG, Anderson SE, Grant I, McNurlan MA, and Garlick PJ.** The determination of low d5-phenylalanine enrichment (0.002–0.09 atom percent excess), after conversion to phenylethylamine, in relation to protein turnover studies by gas chromatography/electron ionization mass spectrometry. *Rapid Commun Mass Spectrom* 6: 421–424, 1992.
8. **Cargile BJ, Bundy JL, Grunden AM, and Stephenson JL Jr.** Synthesis/degradation ratio mass spectrometry for measuring relative dynamic protein turnover. *Anal Chem* 76: 86–97, 2004.
9. **Chen G, Gharib TG, Huang CC, Taylor JM, Misek DE, Kardia SL, Giordano TJ, Iannettoni MD, Orringer MB, Hanash SM, and Beer DG.** Discordant protein and mRNA expression in lung adenocarcinomas. *Mol Cell Proteom* 1: 304–313, 2002.
10. **Conrads TP, Alving K, Veenstra TD, Belov ME, Anderson GA, Anderson DJ, Lipton MS, Pasa-Tolic L, Udseth HR, Chrisler WB, Thrall BD, and Smith RD.** Quantitative analysis of bacterial and mammalian proteomes using a combination of cysteine affinity tags and 15N-metabolic labeling. *Anal Chem* 73: 2132–2139, 2001.
11. **De Feo P, Horber FF, and Haymond MW.** Meal stimulation of albumin synthesis: a significant contributor to whole body protein synthesis in humans. *Am J Physiol Endocrinol Metab* 263: E794–E799, 1992.
12. **Doherty MK, Whitehead C, McCormack H, Gaskell SJ, and Beynon RJ.** Proteome dynamics in complex organisms: using stable isotopes to monitor individual protein turnover rates. *Proteomics* 5: 522–533, 2005.
13. **Fu AZ, Morris JC, Ford GC, and Nair KS.** Sequential purification of human apolipoprotein B-100, albumin, and fibrinogen by immunoaffinity chromatography for measurement of protein synthesis. *Anal Biochem* 247: 228–236, 1997.
14. **Gibson NR, Fereday A, Cox M, Halliday D, Pacy PJ, and Millward DJ.** Influences of dietary energy and protein on leucine kinetics during feeding in healthy adults. *Am J Physiol Endocrinol Metab* 270: E282–E291, 1996.
15. **Greenbaum D, Colangelo C, Williams K, and Gerstein M.** Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol* 4: 117, 2003.
16. **Griffin TJ, Gygi SP, Ideker T, Rist B, Eng J, Hood L, and Aebersold R.** Complementary profiling of gene expression at the transcriptome and proteome levels in Saccharomyces cerevisiae. *Mol Cell Proteom* 1: 323–333, 2002.
17. **Gygi SP, Rist B, and Aebersold R.** Measuring gene expression by quantitative proteome analysis. *Curr Opin Biotechnol* 11: 396–401, 2000.
18. **Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, and Aebersold R.** Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 17: 994–999, 1999.
19. **Gygi SP, Rochon Y, Franza BR, and Aebersold R.** Correlation between protein and mRNA abundance in yeast. *Mol Cell Biol* 19: 1720–1730, 1999.
20. **Halliday D and Ford GC.** Stable Isotopes in Clinical Investigations. In: *Clinical Biochemistry: Principles, Methods, Applications*, edited by Lawson AM. New York: Walter de Gruyter, 1989, p. 684–726.
21. **Hellman U, Wernstedt C, Gonez J, and Heldin CH.** Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing. *Anal Biochem* 224: 451–455, 1995.
22. **Jahoor F, Sivakumar B, Del Rosario M, and Frazer EM.** Isolation of acute-phase proteins from plasma for determination of fractional synthesis rates by a stable isotope tracer technique. *Anal Biochem* 236: 95–100, 1996.
23. **Jensen MD, Kanaley JA, Roust LR, O'Brien PC, Braun JS, Dunn WL, and Wahner HW.** Assessment of body composition with use of dual-energy x-ray absorptiometry: evaluation and comparison with other methods. *Mayo Clin Proc* 68: 867–873, 1993.

24. **Krijgsveld J, Ketting RF, Mahmoudi T, Johansen J, Artal-Sanz M, Verrijzer CP, Plasterk RH, and Heck AJ.** Metabolic labeling of C. elegans and D. melanogaster for quantitative proteomics. *Nat Biotechnol* 21: 927–931, 2003.
25. **Locke S and Figeys D.** Techniques for the optimization of proteomic strategies based on head column stacking capillary electrophoresis. *Anal Chem* 72: 2684–2689, 2000.
26. **Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, and Brown EL.** Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol* 14: 1675–1680, 1996.
27. **Nair KS, Halliday D, and Griggs RC.** Leucine incorporation into mixed skeletal muscle protein in humans. *Am J Physiol Endocrinol Metab* 254: E208–E213, 1988.
28. **Nair KS, Jaleel A, Asmann YW, Short KR, and Raghavakaimal S.** Proteomic research: potential opportunities for clinical and physiological investigators. *Am J Physiol Endocrinol Metab* 286: E863–E874, 2004.
29. **Oda Y, Huang K, Cross FR, Cowburn D, and Chait BT.** Accurate quantitation of protein expression and site-specific phosphorylation. *Proc Natl Acad Sci USA* 96: 6591–6596, 1999.
30. **Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, and Mann M.** Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteom* 1: 376–386, 2002.
31. **Pacy PJ, Read M, and Halliday D.** Influence of insulin on albumin and non-albumin protein fractional synthetic rates in post-absorptive type I diabetic patients. *Eur J Clin Nutr* 44: 343–349, 1990.
32. **Patterson BW, Zhang XJ, Chen Y, Klein S, and Wolfe RR.** Measurement of very low stable isotope enrichments by gas chromatography/mass spectrometry: application to measurement of muscle protein synthesis. *Metab Clin Exper* 46: 943–948, 1997.
33. **Peri S, Navarro JD, Amanchy R, Kristiansen TZ, Jonnalagadda CK, Surendranath V, Niranjan V, Muthusamy B, Gandhi TK, Gronborg M, Ibarrola N, Deshpande N, Shanker K, Shivashankar HN, Rashmi BP, Ramya MA, Zhao Z, Chandrika KN, Padma N, Harsha HC, Yatish AJ, Kavitha MP, Menezes M, Choudhury DR, Suresh S, Ghosh N, Saravana R, Chandran S, Krishna S, Joy M, Anand SK, Madavan V, Joseph A, Wong GW, Schiemann WP, Constantinescu SN, Huang L, Khosravi-Far R, Steen H, Tewari M, Ghaffari S, Blobe GC, Dang CV, Garcia JG, Pevsner J, Jensen ON, Roepstorff P, Deshpande KS, Chinnaiyan AM, Hamosh A, Chakravarti A, and Pandey A.** Development of human protein reference database as an initial platform for approaching systems biology in humans. *Genome Res* 13: 2363–2371, 2003.
34. **Pieper R, Gatlin CL, Makusky AJ, Russo PS, Schatz CR, Miller SS, Su Q, McGrath AM, Estock MA, Parmar PP, Zhao M, Huang ST, Zhou J, Wang F, Esquer-Blasco R, Anderson NL, Taylor J, and Steiner S.** The human serum proteome: display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. *Proteomics* 3: 1345–1364, 2003.
35. **Pratt JM, Petty J, Riba-Garcia I, Robertson DH, Gaskell SJ, Oliver SG, and Beynon RJ.** Dynamics of protein turnover, a missing dimension in proteomics. *Mol Cell Proteom* 1: 579–591, 2002.
36. **Rosenfeld J, Capdevielle J, Guillemot JC, and Ferrara P.** In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. *Anal Biochem* 203: 173–179, 1992.
37. **Walsh MK and Brown RJ.** Use of amino acid analysis for estimating the individual concentrations of proteins in mixtures. *J Chromatogr A* 891: 355–360, 2000.

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