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Sequence Comparison of Rat Liver Phenylalanine Hydroxylase and Its cDNA Clones†

K. J. H. Robson,‡ W. Beattie, R. J. James, R. C. H. Cotton, F. J. Morgan, and S. L. C. Woo*

ABSTRACT: Classical phenylketonuria, an inborn error in metabolism, is caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase. The identification of putative cDNA clones coding for rat liver phenylalanine hydroxylase by hybrid-selected translation has previously been reported [Robson, K. J., Chandra, T., MacGillivray, R. T. A., & Woo, S. L. C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4701–4705]. The authenticity of the clones, however, could not be definitively ascertained at the time because of a lack of amino acid sequence data of the enzyme in the literature. Purified rat liver phenylalanine hydroxylase was subjected to cyanogen bromide treatment, and the resulting fragments were used for N-terminal amino acid sequence analysis. The partial amino acid sequence was then compared to that deduced from an open reading frame in the nucleotide sequence of the cDNA clones. A perfect match of 17 amino acid residues was found between the two sequences following a unique methionine codon present in the nucleotide sequence, thereby providing unambiguous evidence for the identity of the rat liver phenylalanine hydroxylase cDNA clones.

Phenylalanine hydroxylase is a hepatic enzyme in man that catalyzes the oxidation of phenylalanine to tyrosine by utilizing tetrahydrobiopterin as cofactor (Kaufman & Fisher, 1970; Woo et al., 1974; Cotton & Gratter, 1975). Deficiency of this enzymatic activity is the cause of classical phenylketonuria (PKU), which is a genetic disorder that predisposes affected children to development of severe mental retardation (Folling, 1934a,b; Jervis, 1953; Udenfriend & Bessman, 1953; Kaufman, 1976). In patients with untreated PKU, increased serum phenylalanine and its minor metabolites such as phenylpyruvic acid and depletion of serum tyrosine are characteristic (Blau, 1979). The disease is transmitted as an autosomal recessive trait with an average frequency of approximately 1/10 000 among Caucasians, indicating that 2% of the population are carriers of the PKU trait (Thalhammer, 1975; Scriver & Rosenberg, 1973; Bickel et al., 1973).

With the intention of cloning the human phenylalanine hydroxylase gene in order to study the molecular genetics of PKU, we started by cloning the cDNA from rat liver. Phenylalanine hydroxylase mRNA was specifically enriched by polysome immunoprecipitation and used for construction and isolation of the corresponding cDNA clone. Due to the lack of amino acid sequence data of rat liver phenylalanine hydroxylase at the time, identification of the clones necessitated the use of indirect methods such as differential hybridization and hybrid-selected translation (Robson et al., 1982). In this paper, we report the unambiguous verification of the cDNA clones by comparing DNA sequence data with the partial N-terminal amino acid sequence of a cyanogen bromide fragment of purified rat liver phenylalanine hydroxylase.

Materials and Methods

Materials

Restriction enzymes were purchased from Bethesda Research Labs and New England Biolabs. Bacterial alkaline phosphatase was obtained from Worthington Biochemicals and T4 polynucleotide kinase from Boehringer-Mannheim. [γ-^{32}P]ATP (3000 Ci/mmol) and deoxynucleoside [α-^{32}P]triphosphates were purchased from Amersham Corp. Hydrazine was purchased from Pierce Chemical Co. Piperidine (Fisher

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‡ Present address: MRC Unit of Molecular Haematology, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Headington, Oxford OX37DU.

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Scientific) was redistilled prior to use. Acrylamide, bis(acrylamide), and dimethyl sulfate were obtained from Eastman Kodak.

Methods

Purification of Rat Phenylalanine Hydroxylase. The enzyme was extracted from fresh rat liver and partially purified on phenyl-Sepharose (Pharmacia) as described by Shiman (1980). This preparation was then applied to a DEAE-cellulose (Whatman) column (1 × 10 cm) and eluted with a gradient of 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.3, 0.3 M KCl, and 50 mM Tris, pH 7.3. The major peak from the ion-exchange column was then chromatographed on a Sephadex G-100 (Pharmacia) column (2.5 × 150 cm) and eluted with 50 mM NH_4OAc, pH 6.5, and 0.1% sodium dodecyl sulfate (SDS). The peak fractions (OD_{230nm}) were pooled, lyophilized, and redissolved in water, and the protein was precipitated with 4 volumes of acetone. The level of contaminants at all stages of purification was monitored by SDS–polyacrylamide gel electrophoresis (Weber & Osborn, 1969) followed by silver staining (Morrissey, 1981).

Cyanogen Bromide Cleavage and Amino Acid Sequence Analysis. The acetone-precipitated protein was cleaved in 70% formic acid by using a 30-fold molar excess of cyanogen bromide for 16 h at room temperature. The reaction mixture was then diluted with water and lyophilized; the fragments were resuspended in 1% trifluoroacetic acid and loaded directly into the sequencer cup. Automated amino acid sequence determinations were performed by using a Beckman 890C sequencer equipped with a straight-walled cup, using Polybrene as a carrier and 0.1 M Quadrol buffer (Beggs et al., 1978). Thiazolinones were treated with 20% trifluoroacetic acid at 80 °C for 15 min under nitrogen, and the resulting phenylthiohydantoin (PTH)-amino acids were analyzed by high-pressure liquid chromatography (Hewlett-Packard) using the method of Zimmerman et al. (1976).

Sequence Analysis of cDNA Clones. The cDNA inserts coding for phenylalanine hydroxylase were released from the recombinant plasmids prPH 191 and prPH 198 (Robson et al., 1982) by digestion with *Hpa*II or *Hha*I. The cDNA inserts were isolated by preparative polyacrylamide gel electrophoresis at pH 8.2. The purified cDNA insert was then digested with a specific restriction enzyme and labeled at the 5′ end with [γ-^{32}P]ATP using T4 polynucleotide kinase or at the 3′ end with [α-^{32}P]dNTPs using the Klenow fragment of *Escherichia coli* polymerase I. Labeled fragments were recut with an appropriate second restriction enzyme and separated by preparative polyacrylamide gel electrophoresis or strand separated. Sequence analysis was performed as described by Maxam & Gilbert (1977). Computer analysis of the resultant data was carried out by using the programs of Staden (1977).

Results

The isolation of two putative rat liver phenylalanine hydroxylase cDNA clones designated prPH 191 and prPH 198 has been described previously (Robson et al., 1982). These clones were constructed by using standard techniques and were inserted into the *Pst*I site of pBR322 by GC tailing. The size of the corresponding mRNA in rat liver as indicated by Northern blot analysis was predicted to be about 2.5 kilobases (kb). Restriction mapping showed that the two cDNA clones overlap each other considerably and represent only about half of the mRNA molecule. The two cDNA clones were sequenced in their entirety according to the strategy shown in Figure 1. In most cases, both strands of the inserted DNA fragments from either of the two clones were subjected to

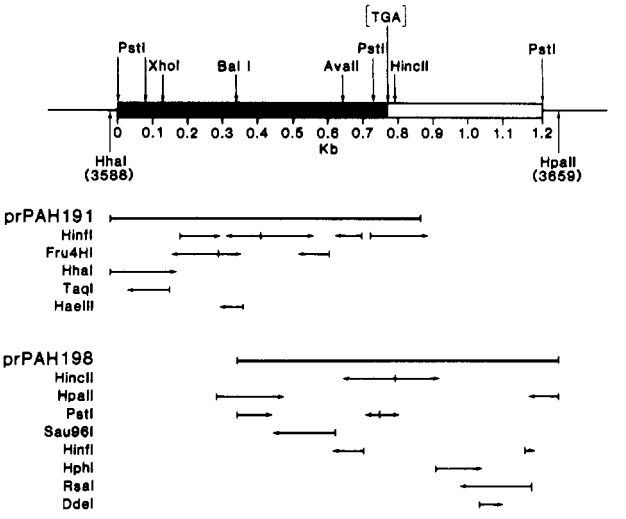

FIGURE 1: Restriction map and sequencing strategy of two rat phenylalanine hydroxylase cDNA clones, prPH 191 and prPH 198. The small vertical lines under the maps for the two cDNA clones represent end-labeling sites, and arrows represent both the direction and extent of DNA sequenced. The *Hha*I (3588) and *Hpa*II (3659) in the composite map represent the restriction sites present in the cloning vector pBR322. The solid bar represents an open reading frame, and the open bar represents a 3′ untranslated region.

sequence analysis. When only one strand was sequenced, at least two independent experiments were performed using different labeling sites. All restriction sites used for labeling and recutting were sequenced across to ensure the accuracy of the nucleotide sequence.

Since the two cDNA clones do not contain the complete coding sequence of the enzyme and there was no poly(A) tract revealed from the DNA sequence, the orientation of the clones relative to protein sequence was not obvious. The DNA sequence was thus subjected to computer analysis, and an open reading frame of 246 amino acids was found, followed by a termination codon UGA and 423 nucleotides of 3′ noncoding sequence (Figure 2). Within the open reading frame sequence, there is only one internal methionine codon which is located in the carboxyl half of the enzyme molecule. Thus, cyanogen bromide cleavage of the corresponding protein should yield a carboxyl-terminal peptide consisting of 177 amino acid residues with an approximate molecular weight of 18 000.

In order to unambiguously verify the authenticity of the cDNA clones to rat liver phenylalanine hydroxylase, it is necessary to determine a partial amino acid sequence of the enzyme which can be compared with the DNA sequence of the clones. Since rat liver phenylalanine hydroxylase contains only one methionine residue per peptide chain (Shiman, 1980), it is possible that the codon for this residue is represented in the cDNA clone. Thus, a strategy to verify the rat cDNA clones was designed by determining the N-terminal amino acid sequence of cyanogen bromide fragments of the purified rat enzyme.

The purity of phenylalanine hydroxylase isolated from rat liver was determined by polyacrylamide gel electrophoresis followed by silver staining and judged to be in excess of 90% (Figure 3A, lane a). Cleavage of the purified enzyme with cyanogen bromide yielded two fragments of 32 000 and 18 000 daltons (Figure 3B, lane b). The fact that the molecular weight sum of the two fragments equals that of the purified enzyme (50 000) is consistent with the amino acid composition data which showed the presence of a single methionine (Shiman, 1980). Since phenylalanine hydroxylase, a cytoplasmic enzyme, has a blocked amino terminus (unpublished

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 His  Ile  Phe  Pro  Leu  Leu  Glu  Lys  Tyr  Cys  Gly  Phe  Arg  Glu  Asp  Asn  Ile  Pro  Gln  Leu
C A C A T T T T C C C A C T T C T G G A A A A G T A C T G C G G T T T C C G T G A A G A C A A C A T T C C A C A G C T G
               48                   58                   68                   78                   88                   98

 Glu  Asp  Val  Ser  Gln  Phe  Leu  Gln  Thr  Cys  Thr  Gly  Phe  Arg  Leu  Arg  Pro  Val  Ala  Gly
G A A G A T G T T T C T C A A T T T C T G C A G A C T T G T A C T G G T T T C C G C C T C C G A C C T G T T G C T G G C
          108                  118                  128                  138                  148                  158

 Leu  Leu  Ser  Ser  Arg  Asp  Phe  Leu  Gly  Gly  Leu  Ala  Phe  Arg  Val  Phe  His  Cys  Thr  Gln
T T A C T G T C A T C T C G A G A T T T C T T G G G T G G C C T A G C C T T C C G A G T C T T C C A C T G C A C A C A G
          168                  178                  188                  198                  208                  218

 Tyr  Ile  Arg  His  Gly  Ser  Lys  Pro  Met  Tyr  Thr  Pro  Glu  Pro  Asp  Ile  Cys  His  Glu  Leu
T A C A T T A G G C A T G G A T C G A A G C C C A T G T A C A C A C C T G A A C C T G A C A T C T G C C A T G A A C T C
          228                  238                  248                  258                  268                  278

 Leu  Gly  His  Val  Pro  Leu  Phe  Ser  Asp  Arg  Ser  Phe  Ala  Gln  Phe  Ser  Gln  Glu  Ile  Gly
T T G G G A C A T G T G C C T T T G T T T T C A G A T C G C A G C T T T G C C C A G T T T T C T C A G G A A A T T G G G
          288                  298                  308                  318                  328                  338

 Leu  Ala  Ser  Leu  Gly  Ala  Pro  Asp  Glu  Tyr  Ile  Glu  Lys  Leu  Ala  Thr  Ile  Tyr  Trp  Phe
C T T G C A T C A C T G G G G G C A C C T G A T G A G T A C A T T G A G A A A C T G G C C A C A A T T T A C T G G T T T
          348                  358                  368                  378                  388                  398

 Thr  Val  Glu  Phe  Gly  Leu  Cys  Lys  Glu  Gly  Asp  Ser  Ile  Lys  Ala  Tyr  Gly  Ala  Gly  Leu
A C T G T G G A G T T T G G G C T T T G C A A G G A A G G A G A T T C T A T A A A G G C A T A T G G T G C T G G G C T C
          408                  418                  428                  438                  448                  458

 Leu  Ser  Ser  Phe  Gly  Glu  Leu  Gln  Tyr  Cys  Leu  Ser  Asp  Lys  Pro  Lys  Leu  Leu  Pro  Leu
T T G T C A T C C T T T G G A G A A T T A C A G T A C T G T T T A T C A G A C A A G C C A A A G C T C C T C C C C C T G
          468                  478                  488                  498                  508                  518

 Glu  Leu  Glu  Lys  Thr  Ala  Cys  Gln  Glu  Tyr  Ser  Val  Thr  Glu  Phe  Gln  Pro  Leu  Tyr  Tyr
G A G C T A G A G A A G A C A G C C T G C C A G G A G T A T T C T G T C A C A G A G T T C C A G C C C T T G T A C T A C
          528                  538                  548                  558                  568                  578

 Val  Ala  Glu  Ser  Phe  Ser  Asp  Ala  Lys  Glu  Lys  Val  Arg  Thr  Phe  Ala  Ala  Thr  Ile  Pro
G T G G C G G A G A G T T T C A G T G A T G C C A A G G A G A A A G T G A G G A C T T T T G C T G C C A C G A T C C C T
          588                  598                  608                  618                  628                  638

 Arg  Pro  Phe  Ser  Val  Arg  Tyr  Asp  Pro  Tyr  Thr  Gln  Arg  Val  Glu  Val  Leu  Asp  Asn  Thr
C G G C C C T T C T C G G T T C G C T A T G A C C C T T A C A C T C A A A G G G T T G A G G T C C T A G A C A A T A C T
          648                  658                  668                  678                  688                  698

 Gln  Gln  Leu  Lys  Ile  Leu  Ala  Asp  Ser  Ile  Asn  Ser  Glu  Val  Gly  Ile  Leu  Cys  Asn  Ala
C A G C A G T T G A A G A T T T T A G C T G A C T C C A T T A A T A G T G A G G T C G G A A T C C T T T G C A A T G C C
          708                  718                  728                  738                  748                  758

 Leu  Gln  Lys  Ile  Lys  Ser  ***
C T G C A G A A G A T A A A G T C G T G A G C A G A A A A T G A C G T C A T G G G C A G A A C T T A G G A G G T C A A C
          768                  778                  788                  798                  808                  818

C A G A A A T C G G T C G A T A G A A G T A T A G T A A C T G C C T T T G T T C C C C T G A A G A A A A A A A A G T T T T
          828                  838                  848                  858                  868                  878

A T T T G A A A C G T C A G C T T T T A T T A T T T T T G C T A A C A C A G T G G A G C A T C A C C A A A T A A A T C A
          888                  898                  908                  918                  928                  938

A A G T T C T C T G A A A T G A A A G T G T G T T A A A A C C C T C T A G G G G T A A A T C T T T C A G A A T C A C A T
          948                  958                  968                  978                  988                  998

T T G A T T T A G A T A A T C T C A T A G C T T C A A T T C G G C T T A A A A T G T A A T T T T C T C A G C T C T C A T
         1008                 1018                 1028                 1038                 1048                 1058

C A A C A T T C A T C A A A T T T G G G G C T T G T G T C A T T G G G G C T T C A T C C G T T C A T T T T T A A C C T C
         1068                 1078                 1088                 1098                 1108                 1118

T C A G G T A A G C T C T G A A G G A A C A C A T T T G G C T G A G T G T A A A A T G T A A G A C T G C C A T C A G G G
         1128                 1138                 1148                 1158                 1168                 1178

A T G A A T C A T T C C G T T C A G T A C A C
         1188                 1198                 1208                 1218                 1228                 1238
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FIGURE 2: DNA sequence of the two overlapping rat phenylalanine hydroxylase cDNA clones and the amino acid sequence deduced from an open reading frame. The first 38 nucleotides not shown in the figure were GC tails and pBR322 DNA sequences. The codon for the unique internal methionine residue is at nucleotides 243–245.

results), the separation of the two fragments prior to amino acid sequence analysis was not necessary. Thus, mixtures of cyanogen bromide fragments were subjected to automated Edman degradation using a Beckman 890C sequencer, and the order of the first 18 amino acid residues is shown in Table I. The partial amino acid sequence of the rat enzyme was then compared with the open reading frame sequence of the cDNA clones following the internal methionine codon at nucleotides 243–245 (Figure 4). The two sequences matched perfectly, providing unambiguous evidence for the authenticity of the rat liver phenylalanine hydroxylase cDNA clones.

Discussion

Phenylalanine hydroxylase purified from rat liver was subjected to cyanogen bromide cleavage, and the fragments were used for N-terminal sequence analysis. A partial amino acid sequence of 17 residues was derived from one of the fragments, which matched perfectly with a segment of the

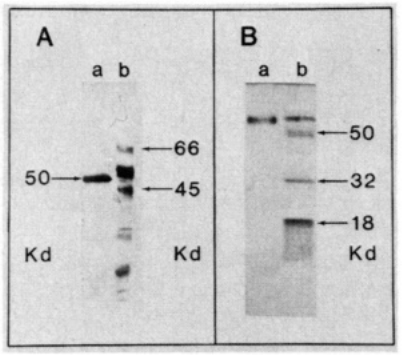

FIGURE 3: Purified rat liver phenylalanine hydroxylase and its cyanogen bromide fragments. (A) SDS–polyacrylamide gel (12.5%) analysis of purified rat phenylalanine hydroxylase used in subsequent structural analysis: lane a, purified enzyme; lane b, molecular weight standards (Sigma). (B) SDS–polyacrylamide gel (15%) analysis of products from cyanogen bromide cleavage of purified rat phenylalanine hydroxylase: lane a, an artificial band generated by loading buffer alone; lane b, cyanogen bromide cleaved enzyme. The residual 50 000-dalton band indicates uncleaved enzyme, and two cyanogen bromide fragments of 32 000 and 18 000 daltons are evident.

```
prPH DNA Sequence: . . . T A C A C A C C T G A A C C T G A C A T C T G C C A T
                        Tyr  Thr  Pro  Glu  Pro  Asp  Ile  Cys  His
                         •    •    •    •    •    •    •         •
Rat PH AA Sequence:     Tyr  Thr  Pro  Glu  Pro  Asp  Ile       His

prPH DNA Sequence:     G A A C T C T T G G G A C A T G T G C C T T T G T T T . . .
                        Glu  Leu  Leu  Gly  His  Val  Pro  Leu  Phe
                         •    •    •    •    •    •    •    •
Rat PH AA Sequence:     Glu  Leu  Leu  Gly  His  Val  Pro  Leu  Phe
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FIGURE 4: Authenticity of the rat phenylalanine hydroxylase cDNA clones. Comparison of the amino acid sequence of a cyanogen bromide fragment of purified rat phenylalanine hydroxylase with that deduced from an open reading frame of the two cDNA clones following the methionine codon.

Table I: Automated Edman Degradation and Amino Acid Sequence Determination of Cyanogen Bromide Fragments of Purified Rat Liver Phenylalanine Hydroxylase[a]

cycle no.	residue	yield (nmol)	cycle no.	residue	yield (nmol)
1	Y	0.68	10	E	1.45
2	T	0.70[b]	11	L	1.24
3	P	1.71	12	L	1.43
4	E	1.76	13	G	0.73
5	P	1.84	14	H	0.70
6	D	1.98	15	V	0.96
7	I	1.78	16	P	1.32
8	X		17	L	0.72
9	H	0.84	18	F	1.25

[a] Approximately 5 nmol of rat phenylalanine hydroxylase cyanogen bromide fragments was sequenced in two separate experiments, each using a different preparation of enzyme. [b] Yield of threonine plus dehydrothreonine.

amino acid sequence deduced from an open reading frame of the putative cDNA clones to rat phenylalanine hydroxylase, providing unambiguous evidence for the identity of these clones. This is particularly important in light of the fact that we had used the rat cDNA clones to identify the corresponding human cDNA clones by cross-hybridization. The human phenylalanine hydroxylase cDNA clones have been used to detect the existence of restriction fragment length polymorphisms in the human genome, which could be applied clinically for prenatal diagnosis and carrier detection in PKU kindreds (Woo et al., 1983). Thus, the definitive authentification of the rat cDNA clones with the protein sequence data also validates the human phenylalanine hydroxylase cDNA clones, which must be documented prior to their clinical applications.

Rat liver phenylalanine hydroxylase activity can be enhanced by phosphorylation using cAMP-dependent protein kinase in vitro in the presence of the natural cofactor tetrahydrobiopterin (Abita et al., 1976). A similar elevation of enzymatic activity had also been observed in rat liver after administration of glucagon in vivo, suggesting that the enzyme is regulated by a phosphorylation–dephosphorylation system (Donlon & Kaufman, 1978). A decapeptide containing the phosphorylation site has previously been isolated and its amino acid sequence determined (Wretborn et al., 1980). A computer search for this amino acid sequence in the two cDNA clones, however, was unsuccessful. Since the cloned DNAs encode only the carboxyl half of the enzyme, the phosphorylation site in the enzyme must reside within the N-terminal half of the molecule.

Tyrosine, the enzymatic product of hepatic phenylalanine hydroxylase, is transported from the liver to the brain and the adrenyl gland where it is converted to dihydroxyphenylalanine (L-DOPA) in the presence of tetrahydrobiopterin by tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis (Blaschko, 1939). Antibodies raised against purified rat liver phenylalanine hydroxylase cross-react with rat adrenyl tyrosine hydroxylase and vice versa (Chikaraishi et al., 1983), suggesting that the two aromatic amino acid hydroxylases are closely related. It is interesting to note that tyrosine hydroxylase is also a phosphoenzyme whose activity can be regulated through a phosphorylation–dephosphorylation system (Meligani et al., 1981). Even though the rat phenylalanine hydroxylase cDNA clones encode only the carboxyl half of the protein molecule which does not contain the phosphorylation region in the enzyme, they still cross-hybridized with a rat adrenyl mRNA species which could be tyrosine hydroxylase mRNA (Robson et al., 1982). If this is indeed the case, it would suggest that there may be significant nucleotide sequence homology between the mRNAs. The molecular cloning of rat tyrosine hydroxylase has recently been reported (Lamouroux et al., 1982; Chikaraishi et al., 1983; Joh et al., 1983). It would be most interesting to compare the amino acid and nucleotide sequences of the two enzymes in order to determine the domains and extents of sequence homology between the two members of this gene family.

Acknowledgments

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Registry No. Phenylalanine hydroxylase, 9029-73-6.

References

Abita, J. P., Milstein, S., Chang, N., & Kaufman, S. (1976) *J. Biol. Chem. 251*, 5310–5314.

Begg, G. S., Pepper, D. S., Chesterman, C. N., & Morgan, F. J. (1978) *Biochemistry 17*, 1739–1744.

Bickel, H., Beckers, R. G., Namberg, E., Schmid-Rüter, E., Feingold, J., Cahalane, S. F., Bottini, E., Jonxis, J. H. P., Colombo, J. P., & Carson, N. (1973) *Acta Paediatr. Scand. 62*, 413–416.

Blaschko, H. (1939) *J. Physiol. (London) 96*, 50–62.

Blau, K. (1979) in *Aromatic Amino Acid Hydroxylase and Mental Disease* (Youdim, M. B. H., Ed.) pp 79–139.

Chikaraishi, D. M., Brilliant, M. H., & Lewis, E. J (1983) *Cold Spring Harbor Symp. Quant. Biol. 48*, 309–318.

Cotton, R. G. H., & Grattan, P. J. (1975) *Eur. J. Biochem. 60*, 427–430.

Donlon, J., & Kaufman, S. (1978) *J. Biol. Chem. 253*, 6657–6659.

Folling, A. (1934a) *Hoppe-Seyler's Z. Physiol. Chem. 227*, 169–176.

Folling, A. (1934b) *Nord. Med. Tidsk. 8*, 1054–1059.

Jervis, G. A. (1953) *Proc. Soc. Exp. Biol. Med. 82*, 514–515.

Joh, T. H., Baetge, E. E., Ross, M. E., & Reis, D. J. (1983) *Cold Spring Harbor Symp. Quant. Biol. 48*, 327–335.
Kaufman, S. (1976) *Adv. Neurochem. 2*, 1–132.
Kaufman, S., & Fisher, D. B. (1970) *J. Biol. Chem. 235*, 4745–4750.
Lamouroux, A., Biguet, N. F., Samolyk, D., Privat, A., Saloman, J. C., Pujol, J. F., & Mallet, J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3881–3885.
Maxam, A., & Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 560–564.
Meligeni, J., Tank, A. W., Stephens, J. K., Dreyer, E., & Weimer, N. (1981) *Cold Spring Harbor Conf. Cell Proliferation 8*, 1377–1385.
Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.
Robson, K. J., Chandra, T., MacGillivray, R. T. A., & Woo, S. L. C. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4701–4705.
Scriver, C. R., & Rosenberg, L. E. (1973) in *Amino Acid Metabolism and Its Disorders*, pp 290–337, Saunders, Philadelphia, PA.
Shiman, R. (1980) *J. Biol. Chem. 255*, 10029–10032.
Staden, R. (1977) *Nucleic Acids Res. 4*, 4037–4051.
Thalhammer, O. (1975) *Humangenetik 30*, 273–286.
Udenfriend, S., & Bessman, S. P. (1953) *J. Biol. Chem. 203*, 961–966.
Weber, K., & Osborne, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Woo, S. L. C. (1983) *Nature (London) 306*, 151–155.
Woo, S. L. C., Gillam, S. S., & Woolf, L. I. (1974) *Biochem. J. 139*, 741.
Wretborn, M., Humble, E., Ragnarssen, U., & Engstrom, L. (1980) *Biochem. Biophys. Res. Commun. 93*, 403–408.
Zimmerman, C. L., Appella, E., & Pisano, J. J. (1976) *Anal. Biochem. 75*, 77–85.

Fluorescence Method for Measuring the Kinetics of Fusion between Biological Membranes†

Dick Hoekstra,* Tiny de Boer, Karin Klappe, and Jan Wilschut

ABSTRACT: An assay is presented that allows continuous and sensitive monitoring of membrane fusion in both artificial *and* biological membrane systems. The method relies upon the relief of fluorescence self-quenching of octadecyl Rhodamine B chloride. When the probe is incorporated into a lipid bilayer at concentrations up to 9 mol % with respect to total lipid, the efficiency of self-quenching is proportional to its surface density. Upon fusion between membranes labeled with the probe and nonlabeled membranes, the decrease in surface density of the fluorophore results in a concomitant, proportional increase in fluorescence intensity, allowing kinetic and quantitative measurements of the fusion process. The kinetics of fusion between phospholipid vesicles monitored with this assay were found to be the same as those determined with a fusion assay based on resonance energy transfer [Struck, D. K., Hoekstra, D., & Pagano, R. E. (1981) *Biochemistry 20*, 4093–4099]. Octadecyl Rhodamine B chloride can be readily inserted into native biological membranes by addition of an ethanolic solution of the probe. Evidence is presented showing that the dilution of the fluorophore, occurring when octadecyl Rhodamine containing influenza virus is mixed with phospholipid vesicles at pH 5.0, but *not* pH 7.4, resulted from virus–vesicle fusion and was not related to processes other than fusion. Furthermore, by use of this method, the kinetics of fusion between Sendai virus and erythrocyte ghosts and virus-induced fusion of ghosts were readily revealed. Dilution of the probe was not observed upon prior treatment of fluorescently labeled Sendai virus with trypsin. Virus-induced fusion between fluorescently tagged ghosts and ghosts devoid of the probe was only observed (at 37 °C) after a low-temperature preincubation; no fluorescence development was seen during virus-induced aggregation at low temperature nor when ghosts and the virus were directly mixed at 37 °C. These results indicated that spontaneous intermembrane transfer of the fluorophore did not occur. It is our contention that this technique may be of considerable value for investigating fusion between biological membranes and, hence, provides an important tool in elucidating the mechanism of fusion in such systems.

As a crucial, intermediate step, membrane fusion is involved in a variety of biological events. It occurs in such diverse processes as intracellular transport, endocytosis (Steinman et al., 1983), and exocytosis (Gratzl et al., 1980), while enveloped viruses exploit their membrane fusion capacity to deliver their genomes into host cells for replication (White et al., 1983). Although many of the physiological functions mediated by fusion events are largely understood, the molecular mechanism by which the fusion process itself occurs is not. To gain insight into this mechanism, various model systems are used, in particular those involving phospholipid vesicles. Studies employing these simplified systems have particularly benefitted from the availability of sensitive and quantitative assays to monitor continuously the fusion reaction (Vanderwerf & Ullman, 1980; Wilschut et al., 1980; Struck et al., 1981; Uster & Deamer, 1981; Hoekstra, 1982a; MacDonald & MacDonald, 1983). Thus, by detailed kinetic analysis of the fusion process it became possible to evaluate the significance of distinct structural and physical membrane changes in relation to the mechanism of fusion (Wilschut et al., 1980, 1981, 1983; Nir et al., 1983; Hoekstra, 1982a,b; Hoekstra & Martin, 1982; Düzgünes et al., 1984).

It would appear highly advantageous to adapt these fusion assays, which monitor either the mixing of contents or the merging of the lipid bilayers, to the more complex biological

† From the Laboratory of Physiological Chemistry, University of Groningen, Bloemsingel 10, 9712 KZ Groningen, The Netherlands. *Received August 7, 1984.* This work was supported in part by NATO Research Grant 151.81.

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