The expression of selected nitrogen fixation (nit) genes from *Klebsiella pneumoniae* in foreign hosts provides an approach to determine the pathway, minimal genetic requirements, and host dependence of nitrogenase assembly. In this study, we investigated the assembly of the αβ2 MoFe protein, responsible for substrate binding and reduction, by introducing *nifD* and *nifK* (encoding respectively, the α and β subunits) into *Escherichia coli* and the yeast *Saccharomyces cerevisiae*. In *E. coli*, both genes were expressed from the *nifHDKY* operon; in yeast, the genes, separately fused to the yeast ΦADH1 promoter, were introduced on two different plasmids. Denaturing immunoblot analyses demonstrated the presence of significant amounts of NiFD and NifK in both hosts. In *E. coli*, the level or perhaps modification of NiFD depended on the growth medium of the bacteria. Nondenaturing, anaerobic immunoblot assays revealed in *E. coli*, nif-specific antigens of lower electrophoretic mobility than Kp1, which may represent assembly intermediates. In yeast, no putative assembled products were evident, and the predominant antigens corresponded to the monomeric forms of the polypeptides. These results indicate that, unlike NifH, the Fe protein subunit (Berns et al., Gershony et al., and Zamir, A. (1985) J. Biol. Chem. 260, 5240–5243), NiFD and NifK are insufficient for the assembly of an electropheretically Kp1-like structure. Homodimerization of nifK and probably of nifD primary gene products does not appear to occur spontaneously and hence is unlikely to represent the initial step in the assembly. The difference between the two hosts suggests that the cellular environment or mode of expression could affect the interaction between the two subunits.

Biological nitrogen fixation, the reduction of molecular nitrogen to ammonia, is catalyzed by nitrogenase. This structurally and functionally unique enzyme (for reviews see Refs. 1 and 2) contains two components: the MoFe protein, responsible for substrate binding and reduction, and the Fe protein, the specific electron donor of the reaction. Both components are multimeric, metallo proteins; the MoFe protein is an αβ2 heterotrameter, whereas the Fe protein is a homodimer. A single iron-sulfur cluster assembled with the Fe protein and several clusters assembled with the MoFe protein belong to several spectroscopically unique types. A FeMo cofactor, associated with the MoFe protein, is considered to play a central role in the catalytic process.

In *Klebsiella pneumoniae*, the genetically best-characterized diazotroph (for reviews see Refs. 3 and 4), at least ten genes are required for the formation of active nitrogenase components. In addition to *nifH* and *nifD*, which encode the single subunit of the Fe protein (Kp2), and the α and β subunits of the MoFe protein (Kp1), respectively, *nifB*, *nifE*, and *V* play a role in the synthesis of the FeMo cofactor, whereas several other genes are assigned processing or modification functions of a nature still not fully understood. Thus, *nifM* was shown to be required for the formation of a functional Fe protein (5, 6), whereas *nifS* and *nifU* were proposed to activate the Fe protein (3) or the MoFe protein (1).

To date, the major approach to the problem of nitrogenase biosynthesis and assembly has been based on functional and structural characterizations of nitrogenase components formed in various *nif* mutants (5, 7). The complete resolution of the *nif* gene cluster of *K. pneumoniae* (4) makes it now possible to apply an alternative approach based on the introduction of selected genes into organisms naturally devoid of *nif* genes. Analyses of the *nif* gene products synthesized in these cells, under suitable conditions, may allow us to determine the minimal genetic requirements and the pathway of assembly of nitrogenase components. Furthermore, this approach may permit us to assess the role, if any, of the host organism in this process.

We have previously used this approach to study the expression and assembly of the Fe protein subunit in two different hosts: *E. coli* and *Saccharomyces cerevisiae* (8, 9). Although no *E. coli* strain is known to fix nitrogen in nature, the functional expression of the *nif* gene cluster from *K. pneumoniae* in *E. coli* (10) indicates that this organism can fully accommodate the formation of active nitrogenase components. *S. cerevisiae* was selected as a representative eukaryote. A nondenaturing immunoblot assay made it possible to detect, in crude cellular extracts, native nitrogenase components, as well as possibly inactive assembly intermediates. This study indicated that in both foreign hosts, NiFH was sufficient for the assembly of a structure exhibiting physical and biochemical resemblance to the Fe protein, although probably still lacking biological activity.

In the present study, we apply a similar approach to study the assembly of the MoFe protein, the more complex of the two nitrogenase components. Earlier studies of *K. pneumoniae nifB* mutants, defective in FeMo cofactor synthesis, indicated the presence of a Kp1-like structure which lacked the cofactor but was probably assembled with some iron-sulfur...
clusters (11, 12). These results suggested that FeMo cofactor was not obligatory for the assembly of α and β subunits into the characteristic αβ₄ tetramer. Consequently, we focused on the two polypeptide subunits of MoFe protein, followed their synthesis, and examined their capability for assembly in both E. coli and yeast.

The results with the two foreign hosts indicate that unlike NiFH, the Fe protein subunit, the constituent polypeptides of the MoFe protein are not assembled to a detectable extent into a structure electrophoretically similar to Kp1. The extracts contained, however, other Kp1-specific antigens which differed between E. coli and yeast; possible assembly intermediates were observed in E. coli, whereas monomeric forms of the subunits were predominant in yeast. Based on the characteristics of the native antigens found in yeast, we propose that homodimers of nadK, and possibly nifD, primary gene products are not intermediates in the assembly of the αβ₄ tetramer complex. Thus, expression of nifD and nadK is insufficient for detection of a Kp1-like structure, and the cellular environment or mode of expression could have an effect on the assembly process.

MATERIALS AND METHODS

Strains and Growth Conditions—Bacterial strains were E. coli MC1061 (13) and wild type K. pneumoniae. E. coli was grown in LB or NFDM (14) containing 50 μg/ml of the L-amino acids: Pro, Glu, Ile, Leu, Ala, and in some cases supplemented with 0.2% ammonium acetate. For selection and growth of transformants, suitable antibiotics were added as follows: 25 μg/ml chloramphenicol; 12 μg/ml tetracycline; 100 μg/ml ampicillin. K. pneumoniae was grown in NFDM supplemented with 0.2% ammonium acetate or in the presence of Na⁺. Procedures for anaerobic growth and for derepression of nitrogenase were as described (9). The yeast strain 733-3A (MATA, his3, leu2) was used throughout this study. Growth was in SD medium supplemented with the required nutrients. Anaerobic growth was as described (8, 9).

Protein Extractions—Exponentially grown E. coli or yeast were extracted aerobically, or anaerobically, essentially as described (9). In some experiments, pelleted bacteria were suspended and boiled in 3% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, 0.06 M Tris-HCl, pH 7.2. The yeast extraction buffer contained 15% glycerol instead of mannitol. Glycerol was also added, to 10% final concentration, to the E. coli and K. pneumoniae lysates.

Gel Electrophoresis and Blotting—Procedures were essentially as described (8, 9). Denaturing, 7.5% polyacrylamide gels were run with different sodium dodecyl sulfate preparations in the electrode buffer: BDH specially pure; Koch-Light; MC & B 95% pure. Electrophoresis was at 35–60 V for 20 h. Nondenaturing 7.5% polyacrylamide gels or 10–25% polyacrylamide gradient gels were made in buffers with 10% glycerol. Electrode buffers contained 5% glycerol. Electrophoresis on uniform gels was at 60 V for 20 h and on gradient gels at 50 V for 70 h. Blots were pretreated and probed with antibodies in solutions containing bovine serum albumin as previously described (8, 9) or in solutions containing 10% sterile non-fat milk and 0.05% Tween 20. Antibodies used were raised against sodium dodecyl sulfate-denatured purified Kp1.

β-Galactosidase Assay—Assays of permeabilized E. coli cells and yeast protein extracts and specific activity definitions were as described (8).

RESULTS AND DISCUSSION

General Approach—Plasmids pJB100 and pZM1 (Fig. 1), used to express nifD and nadK in E. coli, were constructed previously (9). Both plasmids contain the nifHDKY operon and part of nifE cloned in a vector derived from pBR322, whereas pJB100 contains, in addition, another fragment including nadK. Similar to K. pneumoniae, nif gene expression from these plasmids, utilizing the original nif promoters, requires the ntrA and nifA functions (18). Whereas ntrA is present in E. coli, a constitutively expressed nifA is provided by cotransformation with pNR300 (15) or with pDS1, analogous to pNR300 but with the Sall nifA fragment cloned into pBR322 instead of into pACYC184.

Whereas the genetic organization and mode of expression of the two nif genes in E. coli resemble those in K. pneumoniae, in yeast, nifD and nadK coding sequences were separately ligated to yeast regulatory sequences, in pDS7 and pMH1, respectively (Fig. 1). An identical yeast promoter, of the constitutively expressed ADH1 gene (17), was used to express both genes; polyadenylation signals were provided in pDS7.

1 D. Holland, unpublished results.
by the ADH1 terminator sequence and in MH1 presumably
by a sequence within the 2-μm fragment. The two plasmids
also carry different yeast markers to permit selection of
cotransformants. The cloning of nifD and nifK in separate
plasmids permitted us to study the expression of each gene
apart from the other, as well as in combination. To obtain
maximal expression we chose multicopy, autonomously rep-
licating plasmids as vectors. In another approach, only one
of the plasmids, pDS7, could replicate autonomously, whereas
the other plasmid was derived from pMH1 by deletion of the
2-μm sequence, essential for autonomous replication. Cotrans-
formants, probably containing recombinants of the two plas-
mids, expressed somewhat lower levels of NifD and NifK than
cotransformants with pDS7 and pMH1 (data not shown).
Consequently, the latter plasmids were used throughout this
study.

Two methods were used to assess the level of nifD and nifK
products in the foreign hosts. In the first method, crude
protein extracts were resolved by sodium dodecyl sulfate-
polyacrylamide gel electrophoresis followed by immunoblot
analysis with anti-Kpl antibodies. In this analysis, the reso-
lution of NifD and NifK depends on the specific brand of
sodium dodecyl sulfate present in the electrode buffer (20).
With some preparations (e.g. BDH, specially pure) the co-
migration of the two subunits prevents their resolution; with
other preparations (e.g. Koch-Light; MC & B, 95% pure) the
faster mobility of NifD relative to NifK allows the subunits
to separate.

In the second method, we employed E. coli and yeast plas-
mids, analogous to those expressing the intact subunits, but
which contained in-phase lacZ fusions of the relevant nif
genes (Fig. 1). Transformants with these plasmids were as-
sayed for β-galactosidase activity.

In view of the previously reported instability of nitrogenase
denatured K. pneumoniae to air (7), NifD and NifK were analyzed both in
in aerobic and anaerobic E. coli were analyzed for
in aerobic and anaerobic conditions indicated
without or with nitrogenase synthesis. Similar results were previously obtained for the nifH
gene product (8, 9).

nifD and nifK Expression in E. coli—E. coli cotransformants
with pNR300 and pZM1, or with pJB100, were analyzed for
NifD and NifK. pJB100 is identical to pZM1, except for the
presence of nifM. The results (Fig. 2A) show the presence, in
both pJB100 and pZM1 transformants, of two antigens exhib-
ting a similar electrophoretic mobility to the major Kpl
antigens present in wild type K. pneumoniae, derepressed for
nitrogenase synthesis. The latter antigens also fully corre-
spond in mobility to the two bands yielded by purified Kpl
(Fig. 2C) and identified as the α and β subunits of the MoFe
protein (19).

The analysis also shows similar levels of antigen in both
types of transformant. Hence, unlike its effect on NifH (9),
nifM does not appear to influence the level of NifD and NifK.
These results are in agreement with the presence of normal
amounts of MoFe protein subunits in K. pneumoniae nifM
mutants (5) and support the conclusion that the nifM product
does not exert a general effect on transcription from the
nifHDKY promoter.

Although similar in electrophoretic mobility, the Kpl-spe-
cific antigens observed in the LB grown *E. coli* transformants differ in their relative intensity from the corresponding antigens in the *K. pneumoniae* crude extract or in purified Kp1. In the latter two samples, NifD is stained more intensively than NifK, whereas in *E. coli*, NifK is the more intense of the two antigens.

The peculiar intensity ratio observed in *E. coli* transformants could be due to nonspecific interference in blotting or in antibody binding by some host proteins. This possibility was ruled out by analyzing *K. pneumoniae* extracts mixed with extracts of nontransformed *E. coli*. These mixtures exhibited intensity ratios typical of *K. pneumoniae* (data not shown). Furthermore, as shown in Fig. 2B, *E. coli* transformants with pWK220 (20), a plasmid bearing the entire *K. pneumoniae* nif cluster, and grown in NFDM in the presence of N2 exhibited a similar intensity ratio to *K. pneumoniae*. (Fig. 2B includes data from three different experiments and hence the differences in extent of band separation.)

Whereas obviously containing fewer nif genes, the *E. coli* transformants with pZM1 or pPB100 also differed from the pWK220 transformants by having been grown aerobically in LB medium. *E. coli* transformants with pPB100 were therefore analyzed after anaerobic growth in LB or after growth in suitably supplemented NFDM. The results reveal no effect of anaerobiosis but show a similar intensity with NFDM grown cells (Fig. 2B) to that observed with *K. pneumoniae*. Since this pattern remained unaltered on omission of Fe\(^{3+}\) or molybdate from the NFDM medium (data not shown), we are inclined to conclude that it is the growth in poor or rich medium which determines the intensity of the nifD product in immunoblots.

It is possible that NifD intensity in immunoblots does not always reflect its actual quantity. In contrast to antibody staining, resolved α and β subunits from purified Kp1 are stained to a similar degree with Coomassie Blue (Fig. 2C). Furthermore, in yeast (see below), the signal intensity particularly of NifD is drastically reduced when electrophoresis is performed with resolving as compared to nonresolving sodium dodecyl sulfate (Fig. 3).

To obtain an independent estimate of the relative efficiency of nifD and nifK expression, β-galactosidase assays were performed with transformants with pHDI4 or with pHDK14, plasmids analogous to pZM1, which contained nifD'-lacZ, or nifK'-lacZ fusions, respectively. The transformants contained pNR300 to activate transcription from the nifHDKY promoter. In view of the different NifD to NifK ratios observed in cells grown in LB or NFDM, both media were also used to grow the nif'-lacZ containing transformants. The results (Table I) did not indicate any growth-medium dependent variations in β-galactosidase activity. In both media, the more proximal nifD'-lacZ was expressed approximately 4-fold more efficiently than the more distal nifK'-lacZ, a ratio somewhat surprising in view of the equal proportion of α and β subunits in MoFe protein.

These results indicate that the differences in immunoblot intensity of NifD probably do not reflect transcriptional or translational variations. Rather, the differences could reflect a lower stability of NifD in LB as compared to NFDM grown cells. Alternatively, the differences could be the result of some structural modification of NifD which influences its blotting or antibody binding efficiency. Such structural modification could, in principle, be affected by nifH or nifY, present in both pZM1 and pMH1, or by a host function, perhaps common to *E. coli* and *K. pneumoniae*, which is differentially regulated in poor or rich medium. In this respect, it is noteworthy that two forms of NifD differing in their isoelectric points were observed in an earlier analysis of wild type *K. pneumoniae* extracts (5).

In *K. pneumoniae*, genetic complementation analyses and estimations of nif protein levels in various nif mutants indicate intricate regulatory or mutually stabilizing interactions among nitrogenase polypeptides (5). Based on these observations, at least partial assembly has been proposed to be required to protect NifD and NifK from intracellular degradation. Whereas differences in nif gene copy number and mode of expression exist between *E. coli* transformants and wild type *K. pneumoniae*, it is nevertheless of interest to observe similar levels of NifD and NifK in *E. coli* and in *K. pneumoniae* derepressed by growth in aspartate.\(^2\) These results suggest that the absence in *E. coli* of additional nif functions such as nifB, nifS (5), and nifU could have a lesser effect on NifD and NifK stability than it has in *K. pneumoniae*. Consequently, *K. pneumoniae* could have evolved a specific mechanism for the degradation of nonfunctional nitrogenase polypeptides (7). Such mechanisms may not be common to all N\(_2\)-fixing organisms, as recently demonstrated.

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\(^2\) D. Govezensky, unpublished results.
Characteristics of MoFe Protein Subunits Expressed in Foreign Hosts

### Table I

| Assay of β-galactosidase expressed from different nif-lacZ fusions in E. coli and yeast transformants |
|---|
| **Host** | **nif plasmid** | **Relevant genes** | **Specific activity units** |
|---|---|---|---|
| E. coli | pHD14 | nif H-nif D'-lacZ | 2745 (LB)* 3150 (NFDM)* |
| E. coli | pHDK14 | nif H-nif D'-nif K'-lacZ | 762 (LB)* 882 (NFDM)* |
| Yeast | pDS19 | nif D'-lacZ | 1331* |
| Yeast | pDS19/pMH1 | nif D'-lacZ | 794* |
| Yeast | pMH1Z | nif K'-lacZ | 518* |
| Yeast | pMH1Z/pDS7 | nif K'-lacZ/nif D | 540* |

* Specific activity measured in permeabilized cells, grown in the medium indicated in parentheses.

**FIG. 4.** Nondenaturing immunoblot analysis of E. coli transformants. E. coli transformants were grown in NFDM with amino acids, extracted, and fractionated under anaerobic conditions. Aliquots corresponding to 10^8 cells were analyzed in each lane. Lane 1, nontransformed E. coli; lane 2, E. coli transformed with pJB100 and pNR300; lane 3, E. coli transformed with pWK220 and pDS1; Kp, derepressed K. pneumoniae extract.

for nif-deletion mutants of Azotobacter vinelandii (21).

**nifD and nifK Expression in Yeast—**Immunoblot analysis of yeast transformed with pDS7 or pMH1 or cotransformed with these two plasmids is shown in Fig. 3. With nonresolving sodium dodecyl sulfate yeast transformed separately with pDS7 or pMH1 contain co-migrating antigens of comparable intensity, corresponding to the major Kp1 antigen in the K. pneumoniae extracts. Under resolving conditions, nif antigens in pDS7 and pMH1 transformants correspond, respectively, to the faster and slower migrating antigens evident in the K. pneumoniae extract. The cotransformants contain both nif antigens. These results demonstrate the co-expression of nifD and nifK in yeast and corroborate the previous identification of these two bands as the α and β subunits of the MoFe protein (19).

A quantitative comparison between the patterns shown in Fig. 3, A and B, indicates similar signal intensities for NifD and NifK with the nonresolving sodium dodecyl sulfate, but a drastically reduced intensity of NifD under resolving conditions. Thus, the weak NifD signal can be attributed, at least partly, to the effect of the resolving sodium dodecyl sulfate. Unlike E. coli, alteration in the growth conditions, e.g. addition of molybdate to the medium, had no effect on the intensity pattern (data not shown).

When extracts of separate transformants with pDS7 and pMH1 were mixed in equal proportions, the relative staining intensity of NifD and NifK was similar to that observed in the cotransformants. These results indicate that the steady-state levels of the nif antigens in the cotransformants are similar to those in the separate transformants, although the sensitivity of the assay is insufficient to reveal some variation between the two.

The relative efficiency of nifD and nifK expression in yeast was also evaluated by the use of pDS19 and pMH1Z analogs, respectively, of pDS7 and pMH1 but containing in-phase lacZ fusions instead of the intact nif genes. These plasmids were transformed into yeast individually or together with plasmids expressing the second, intact nif gene. Enzyme assays of the transformed yeast (Table I) show a 2-3 fold higher β-galactosidase level in pDS19 as compared to pMH1Z transformants. When cotransformed with pMH1, the level of β-galactosidase expressed from pDS19 drops by approximately 40%, whereas in the reciprocal combination, pDS7 has only a slight effect on β-galactosidase expressed from pMH1Z. The basis for the different levels of nifD and nifK expression or for the interference observed in cotransformants is not known.

Thus, in both hosts nifD appears to be expressed more efficiently than nifK, although this difference may not be reflected in the steady-state levels of the intact gene products.

**Assembly Analysis—**In order to examine the native forms of the nif gene products in the foreign hosts, extracts of anaerobically grown E. coli or S. cerevisiae transformants were prepared and fractionated under anaerobic, nondenaturing conditions and subsequently analyzed on immunoblots.

With E. coli, we first established that the nature of the host and mode of expression of the nif genes and growth conditions did not affect the electrophoretic behavior of Kp1. For this purpose E. coli was cotransformed with pWK220, containing the entire nif cluster and pDS1, a compatible plasmid containing a constitutively expressed nifA. These cotransformants were grown under identical conditions to E. coli cotransformants with pJB100 and pNR300. The two types of E. coli transformants were compared to K. pneumoniae derepressed for nitrogenase synthesis (Fig. 4). The major Kp1-specific antigen in the K. pneumoniae extract represents native Kp1 as shown by 55Fe labeling (9) as well as by molecular weight estimation (Fig. 5). Additional, more slowly migrating bands may represent oxidized or other forms of the protein. The pattern observed with the pWK220 transformed E. coli closely resembles that of K. pneumoniae indicating that in this case the nature of the host, mode of nif gene expression, or growth conditions do not affect the electrophoretic behavior of Kp1.
FIG. 5. Non-denaturing immuno-blot analysis of yeast transformants. Yeast were grown, extracted, and electrophoresed under anaerobic non-denaturing conditions. A, aliquots corresponding to 2 × 10⁶ cells were electrophoresed on a 7.5% polyacrylamide gel (experiment 1). Kp, derepressed K. pneumoniae extract; KD, cotransformants with pMH1 and pDS7 transformants; Host, nontransformed yeast. B, the same extracts as analyzed in A (experiment 2). Kp, derepressed K. pneumoniae extract; lane 1, cotransformants with pMH1 and pDS7; lane 2, nontransformed yeast. C, the same extracts as analyzed in B, but electrophoresed under denaturing conditions with resolving sodium dodecyl sulfate. Lane description as in B. D, aliquots corresponding to 5 × 10⁶ cells were electrophoresed on a 10–25% gradient polyacrylamide gel. Kp, derepressed K. pneumoniae extract; KD, cotransformants with pMH1 and pDS7; D, pDS7 transformants; Host, nontransformed yeast; K, pMH1 transformants; 67, 232, position of molecular weight markers: bovine serum albumin, 67 KDa, catalase, 232 KDa.

ends. In other experiments, somewhat more distinct bands could be discerned. This electrophoretic behavior suggests that the nif material in E. coli is quite unstable and is easily degraded or aggregated. These unstable forms could represent intermediates in the assembly of Kp1 which accumulate in the absence of additional nif functions. The characteristics of these putative intermediates cannot be attributed solely to the lack of the FeMo cofactor: K. pneumoniae nifB⁻ mutants, defective in FeMo-cofactor biosynthesis, (11, 12), contain a well-defined nif antigen co-migrating with native Kp1 (data not shown).

E. coli also contains some minor nif antigens which migrate faster than native Kp1. These fast-migrating weak bands could represent monomeric forms of the nif proteins, as discussed below. E. coli transformants grown in NFDM exhibited essentially the same pattern as bacteria grown in LB.

In yeast, nifD and nifK were introduced on different plasmids, which made it possible to analyze separately the native forms of NifD and NifK, as well as to identify co-assembled products in cotransformants. Yeast transformed with nifD or nifK or cotransformed with both genes were also analyzed both under resolving and nonresolving denaturing conditions. In the experiment shown in Fig. 5A, yeast transformed with nifK alone or with nifK and nifD contained native nif antigens which migrated faster than native Kp1, a faint band which migrated similarly to Kp1, and several other diffuse nif antigens which migrated more slowly than Kp1. In transformants with nifD alone, no distinct bands were evident but only a trail of slow-migrating material. The analysis under denaturing conditions, with nonresolving sodium dodecyl sulfate (not shown), gave results similar to those shown in Fig. 5A. The band corresponding to both NifD and NifK was more intense in cotransformants than in the individual transformants. In another experiment, the same extracts as those analyzed for native nif gene products (Fig. 5B) were also analyzed by electrophoresis with resolving-type sodium dodecyl sulfate (Fig. 5C). These analyses show again that although cotransformants express both nifD and nifK, they do not contain nif antigens other than those observed in transformants with nifK alone. Thus, these analyses do not provide evidence for any interaction between the nif polypeptides in yeast cells.

To further characterize the non-denatured nif antigens in yeast, similar extracts to those analyzed in Fig. 5A were resolved by prolonged electrophoresis on a 10–25% polyacrylamide gradient gel (Fig. 5D). Under these conditions, the sieving effect of the gel is a predominant factor in the separation, and the extent of migration of the proteins permits an approximate estimation of their molecular mass. The similar migration of Kp1 (1) and the 232-kDa catalase marker is consistent with this expectation. A distinct nif-specific antigen in pMH1 transformants migrates similarly to the 67-kDa bovine serum albumin marker. This antigen most likely corresponds to the 57,751-kDa NifK monomer which has not yet reached its equilibrium position on the gel. A ladder of more slowly migrating, increasingly fainter bands could represent aggregated or -S-S-cross-bridged forms of NifK. The monomeric form disappears when the extracts are flushed with air prior to electrophoresis (not shown). Transformants with pDS7 contain several, relatively faint nif antigens. A distinct, although weak, antigen migrating somewhat faster than NifK could represent the NifD monomer, whereas more slowly moving bands probably represent aggregated forms of the protein. This result shows that although a significant amount of NifD is present in the extract (Fig. 3), very little of this protein is evident under non-denaturing conditions. A tendency to aggregate or extreme oxygen sensitivity could have caused NifD to form structures too large to enter the gel.
The pattern seen with the cotransformants appears to be a composite of the patterns seen with the single transformants. Hence, there is no evidence for an interaction between the two \textit{nif} proteins in yeast or for extensive homodimerization. The latter observation suggests that homodimers of \textit{nifK}, and possibly of \textit{nifD}, primary gene products are not formed spontaneously and hence may not constitute intermediates in MoFe protein assembly.

Thus, no Kpl-like structure is evident in either host, but each contains different forms of nondenatured \textit{nif} antigens. The basis for this difference could be manifold. The two hosts obviously vary in their intracellular environments, as well as in the concentration of \textit{nif} polypeptides. A specific host effect is suggested by the behavior of the \textit{nifD} product. Rough estimates based on \(\beta\)-galactosidase assays indicate several fold higher levels of both NiFD and NiFK in \textit{E. coli}, as compared to yeast. A significant accumulation of nitrogenase polypeptides might be a prerequisite for assembly, as suggested by the long lag observed between the onset of \textit{nif} gene expression and the start of substrate reduction in derepressed \textit{K. pneumoniae} (22). Differences in the mode of \textit{nif} gene expression between the two organisms could also be of significance. In \textit{E. coli}, the presence of both genes in the same operon may lead to local accumulation of the two products and may also allow them to interact, even before translation is completed.

In this respect, it is intriguing to note that in all \(N_{2}\)-fixing organisms analyzed to date, \textit{nifD} and \textit{nifK} are present on the same operon (23). However, co-expression of the two polypeptides may not be obligatory for assembly, since in \textit{A. vinelandii}, functional MoFe protein was reconstituted by mixing extracts of strains carrying \textit{nifD} or \textit{nifK} deletions (21).

Based on our previous and present analyses we propose the following assembly pathways for Fe protein and MoFe protein. Regardless of the nature of the host, NiFH, the Fe protein subunit dimerizes into a structure similar by several criteria to native Kp2. This spontaneously formed, dimeric structure is the likely substrate for subsequent modification or activation reactions. In contrast, the MoFe protein subunits do not assemble spontaneously into homodimers, and the interaction between the two subunits could occur under some conditions but not under others. Modification may take place already at the monomer level and could strongly affect the assembly process or the structural characteristics of the assembled product. The modification may involve the assembly of iron-sulfur clusters, catalyzed perhaps by \textit{nifS} and \textit{nifU} (1). An extension of the approach detailed here provides a means to identify the additional \textit{nif} genes required for assembly and to delineate their functions.

Acknowledgments—We are grateful to R. R. Eady for purified Kpl and G. P. Roberts for \textit{K. pneumoniae} \textit{nif} mutants (both are from the Dept. of Bacteriology, College of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, WI).

REFERENCES

1. Orme-Johnson, W. H. (1985) \textit{Annu. Rev. Biophys. Biophys. Chem.} \textbf{14}, 419-469
2. Burgess, B. K. (1985) in \textit{Nitrogen Fixation Research Progress} (Evans, H. J., Bottomley, P. J., and Newton, W. E., eds) pp. 543-549, Martinus Nijhoff, Dordrecht, Netherlands
3. Brooks, S. J., Imperial, J., and Brill, W. J. (1985) in \textit{Nitrogen Fixation and CO\textsubscript{2} Metabolism} (Ludin, P. W., and Burris, J. E., eds) pp. 65-74, Elsevier, New York.
4. Cannon, F., Benyon, J., Buchanan-Wollaston, V., Burghoff, R., Cannon, M., Kwiatkowsky, R., Laver, G., and Rubin, R. (1985) in \textit{Nitrogen Fixation Research Progress} (Evans, H. J., Bottomley, P. J., and Newton, W. E., eds) pp. 453-460, Martinus Nijhoff, Dordrecht, Netherlands
5. Roberts, G. P., MacNeil, T., MacNeil, D., and Brill, W. J. (1978) \textit{J. Bacteriol.} \textbf{136}, 267-279
6. Howard, K. S., McLean, P. A., Hansen, F. B., Lemley, P. V., Koblan, K. S., and Orme-Johnson, W. H. (1986) \textit{J. Biol. Chem.} \textbf{261}, 772-778
7. Roberts, G. P., and Brill, W. J. (1980) \textit{J. Bacteriol.} \textbf{144}, 210-216
8. Berman, J., Zilberstein, A., Salomon, D., and Zamir, A. (1985) \textit{Gene} (Amst.) \textbf{35}, 1-9
9. Berman, J., Gereshoni, J. M., and Zamir, A. (1985) \textit{J. Biol. Chem.} \textbf{260}, 5240-5243
10. Dixon, R. A., and Postgate, J. R. (1972) \textit{Nature} \textbf{237}, 102-103
11. Hawkes, T. R., and Smith, B. E. (1983) \textit{Biochem. J.} \textbf{209}, 43-50
12. Shah, V. K., Ugalde, R. A., Imperial, J., and Brill, W. J. (1984) \textit{Annu. Rev. Biochem.} \textbf{53}, 231-257
13. Casadaban, M. J., Martinez-Arias, A., Shapira, S. K., and Chou, J. (1985) \textit{Methods Enzymol.} \textbf{100}, 293-308
14. Dixon, R., Kennedy, C., Kondorosi, A., Krishnapillai, V., and Merrick, M. (1977) \textit{Mol. Gen. Genet.} \textbf{157}, 189-198
15. Bitoun, R., Berman, J., Zilberstein, A., Holland, D., Cohen, J. B., Givol, D., and Zamir, A. (1983) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{80}, 5812-5816
16. Scott, K. F., Rolfe, B. G., and Shine, J. B. (1981) \textit{J. Mol. Appl. Genet.} \textbf{1}, 71-81
17. Ammerger, G. (1983) \textit{Methods Enzymol.} \textbf{101}, 192-201
18. Lers, A., Bitoun, R., and Zamir, A. (1986) \textit{J. Bacteriol.} \textbf{165}, 175-180
19. Kennedy, C., Eady, R. R., Kondorosi, E., and Rekosh, D. K. (1976) \textit{Biochem. J.} \textbf{155}, 383-389
20. Puhler, A., Burkhardt, H. J., and Klipp, W. (1979) \textit{Mol. Gen. Genet.} \textbf{176}, 17-24
21. Robinson, A. C., Burgess, B. K., and Dean, D. R. (1986) \textit{J. Bacteriol.} \textbf{166}, 190-196
22. Cannon, M., Hill, S., Kavanaugh, E., and Cannon, F. (1985) \textit{Mol. Gen. Genet.} \textbf{198}, 198-206
23. Evans, H. J., Bottomley, P. J., and Newton, W. E. (eds) (1985) see reviews in \textit{Nitrogen Fixation Research Progress}, Martinus Nijhoff, Dordrecht, Netherlands