Nucleotide–Iron-Sulfur Cluster Signal Transduction in the Nitrogenase Iron-Protein: The Role of Asp^{125}

Dana Wolle, Dennis R. Dean, James Bryant Howard*

Electron transfer in nitrogenase involves a gating process initiated by MgATP (magnesium adenosine triphosphate) binding to Fe-protein. The redox site, an 4Fe:4S cluster, is structurally separated from the MgATP binding site. For MgATP hydrolysis to be coupled to electron transfer, a signal transduction mechanism is proposed that is similar to that in guanosine triphosphatase proteins. Based on the three-dimensional structure of Fe-protein, Asp^{125} is likely to be part of a putative transduction path. Altered Fe-protein with Glu replacing Asp has been prepared and retains the ability for the initial nucleotide-dependent conformational change. However, either MgADP or MgATP can induce the shift and Mg binding to the nucleotide is no longer essential.

The nitrogenase complex is the bacterial enzyme system for the reduction of dinitrogen gas to ammonia. The enzyme is a reversible complex of two metalloproteins, the MoFe-protein and the Fe-protein. Whereas the MoFe-protein contains the site of substrate reduction, the Fe-protein is the exclusive electron donor for the reaction (*1*, *2*). The distinctive role of the Fe-protein stems from the necessity for quasi-unidirectional electron transfer between components in order for the MoFe-protein to accumulate the multiple number of electrons needed to reduce substrates. Electron transfer is coordinated by the hydrolysis of two Fe-protein–bound MgATP molecules (*2*) in an elegant molecular switching or "gating" mechanism. Because MgATP hydrolysis occurs only when the complex is formed, nonproductive electron transfer is avoided. Although structural details of the gating process are not known, there is ample physiochemical evidence for different, nucleotide-dependent conformational states in the Fe-protein (*2–4*). One dramatic manifestation of two conformational states is the susceptibility of the redox-active 4Fe:4S cluster to chelators. The cluster is exposed and can be chelated only when MgATP is bound; the cluster is sequestered and chelation is inhibited by MgADP (*4*). Apparently, electron transfer occurs during the switching between these two states [nonhydrolyzable ATP analogs induce Fe chelation but do not support substrate reduction (*4*)].

The new x-ray diffraction structure for Av2-WT (wild-type *Azotobacter vinelandii* Fe-protein) allows one to consider potential gating mechanisms (*5*). As had been suspected, the nucleotide binding sites include the "Walker type-A and type-B" primary sequences (*6*), residues 9 to 16 and 125 to 128 of Av2-WT (*7*). Likewise, the single 4Fe:4S cluster, the site of electron transfer, is located at the interface between the two identical Av2-WT subunits and is symmetrically liganded by the two pairs of cysteines, Cys^{97} and Cys^{132} (*8*). Most significantly, the nucleotide binding domain is well separated from the bridging cluster, and for events at the nucleotide site to be expressed at the cluster, conformational changes must occur (*3–5*).

The crystal structure of Av2-WT reveals substantial homology in the secondary structures of Av2-WT and the guanosine triphosphatase (GTPase), $p21^{ras}$ (*5*, *9*). In addition, nitrogenase and the G-protein family have in common the use of nucleotide hydrolysis as a kinetic mechanism to temporally switch between two conformations (*2*, *9*). The similarity of their structures may lead to their function as switching enzymes. In the case of nitrogenase, electron transfer occurs during the conformational change that serves as the opening and closing of the pathway (*10*), which results in the essentially unidirectional, intermolecular electron transfer. Another common feature is that nucleotide hydrolysis occurs in association with a second component, MoFe-protein for nitrogenase or GAP (GTPase-activating protein) for G proteins. A number of functionally important residues are also conserved, such as Lys^{15}, Ser^{16}, and Asp^{125} of the Av2-WT sequence (*11*). We investigated the role of Asp^{125} because the analogous residue in $p21^{ras}$ (Asp^{57}) is a ligand of the nucleotide-bound Mg and appears to participate in transmitting a conformational change upon nucleotide hydrolysis. Indeed, in $p21^{ras}$ the largest α-carbon movement is 7 to 11 residues down from Asp^{57} (*9*), or by extrapolation to Av2-WT, conformational changes transmitted by Asp^{125} would include the cluster ligand Cys^{132}.

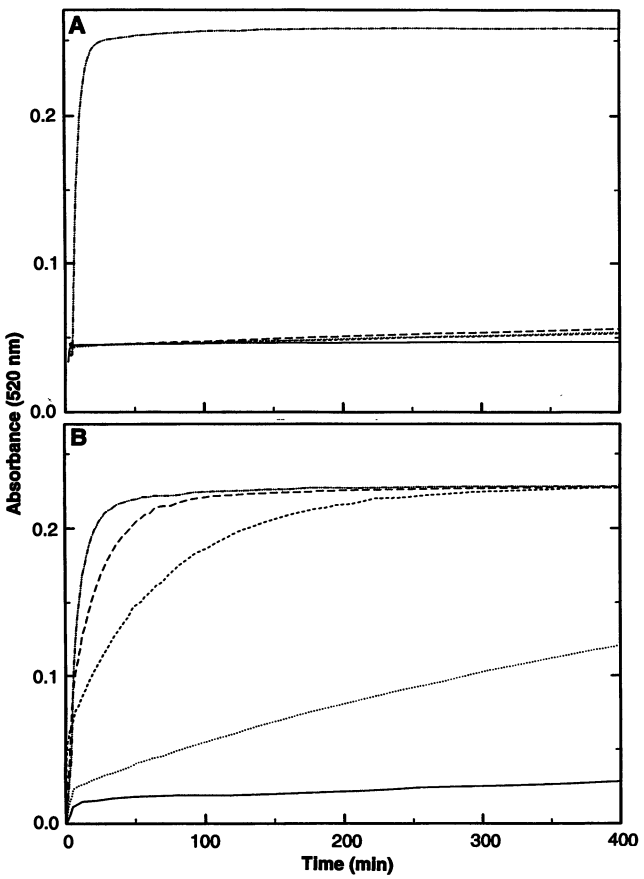

Fig. 1. Chelation of Fe with α-α′ bipyridyl from (**A**) Av2-WT and (**B**) Av2-D125E. Anaerobic quartz cuvettes contained 50 mM tris buffer, pH 8.0, 15 mM $Na_2S_2O_4$, 7.5 μM Av2-WT or 7.6 μM Av2-D125E, ±10 mM $MgCl_2$, and 4 mM nucleotide. Chelation reactions were initiated by the addition of 40 mM α-α′ bipyridyl stock solution (2 mM final concentration) and were monitored by the increase in absorbance at 520 nm due to the Fe(α-α′ bipyridyl)$_3$ complex. (A) (——); no added nucleotide; (- - - -), + ADP; (·····), + ATP; (– –), + MgADP; and (·–·–·), + MgATP. (B) (——), no nucleotide added; (·····), + MgADP; (- - - - -), + ADP; (– –), + ATP; and (·–·–·), + MgATP.

D. Wolle and J. B. Howard, Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455. D. R. Dean, Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

*To whom correspondence should be addressed.

To investigate the role of Asp^{125} in the gating process, an altered Av2 with a glutamic acid substitution at position 125, designated Av2-D125E, was generated by site-directed mutagenesis (GAC → GAA), genomic recombination, and selection protocols we have described previously (*8, 12*). The resulting mutant *A. vinelandii* strain, DJ-576, was incapable of diazotrophic growth and had no whole cell–acetylene reduction activity. Av2-D125E was isolated and characterized as described for other altered Av2 (*12*). As expected from the whole-cell studies, no substrate reduction activity or ATP hydrolysis was detected even when assay conditions modified for observing low and altered activity levels were used (*12, 13*). It should be noted that Av1 (MoFe-protein from *A. vinelandii*) isolated from DJ-576 was fully active. Because Fe-protein participates in the biosynthesis of FeMo-cofactor as well as in substrate reduction (*14*), for the Av1 to be active, Av2-D125E must still function in FeMo-cofactor biosynthesis. This result implies that MgATP hydrolysis is not required in this connection.

In the $p21^{ras}$-MgGTP complex, Asp^{57} indirectly coordinates Mg by way of an intervening water molecule (*9*). Upon hydrolysis of the GTP, Asp^{57} shifts to direct coordination of MgGDP with an ensuing adjustment in the conformation. Hence, one rationale for substituting Glu for Asp in Av2-WT was that the extra methylene group might occupy the space normally held by the hydroxyl in the analogous $p21^{ras}$ Mg-water-carboxylate ligand. To this end, the ability of various nucleotides to induce the conformational changes associated with "exposure" of the cluster to chelators was investigated with Av2-D125E. As shown in Fig. 1A, the cluster in Av2-WT is chelated by α,α′-bipyridyl only when MgATP is present. In striking contrast, the cluster is readily chelated from Av2-D125E in the presence of either MgATP or MgADP (Fig. 1B). Although there is a slow rate of chelation in the absence of nucleotide, the rate is far slower than the chelation of protein inactivated by oxygen, heat, or pH treatment. Most importantly, Av2-D125E has retained the nucleotide dependence of the chelation reaction, but now MgADP is capable of inducing the necessary conformational change. The loss of nucleotide specificity is not general because MgAMP or MgGTP are ineffective (Fig. 1B).

Our design rationale anticipated that MgADP might be able to induce a conformational change in Av2-D125E; what was unexpected was the change in Mg requirement. Metal-free nucleotides are also fully able to induce conformational changes in Av2-D125E that allow the cluster to be chelated (Fig. 1B). Indeed, the rate of chelation is faster for ADP than for MgADP, suggesting that ADP more closely mimics MgATP. The possibility that trace metals might be affecting our results was tested by using EDTA, which effectively removes most metals from nucleotides. The cluster is chelated by EDTA only from Av2-D125E and only if ATP or ADP is present (Fig. 2).

The difference between Av2-WT and Av2-D125E is also evident in direct ADP binding measurements. In spite of experimental variation inherent in working with these extremely oxygen sensitive proteins, there is a significant change in the number of MgADP binding sites in Av2-D125E in the concentration range of the nucleotide used in our studies (*15*). As shown in Fig. 3, the best fit of the binding data is for approximately two sites in Av2-D125E versus one site in Av2-WT, although the apparent dissociation constants (K_D's) are similar. Two binding sites for Mg-free ADP were also found in Av2-D125E (Table 1), whereas no ADP binding could be detected for Av2-WT. In the Av2-WT crystal structure, there is a partial occupancy for one ADP (*5*). It is not clear at present whether there is a metal associated with this ADP (*16*) or whether it represents a tightly bound ADP (with without associated metal) that does not readily exchange with solution ADP.

The effects of the Glu substitution for Asp^{125} can be evaluated in light of the new x-ray structure (*5*). An abbreviated view of the nucleotide binding site in Av2-WT is shown in Fig. 4. As pointed out by Georgiadis *et al.* (*5*), there are two potential

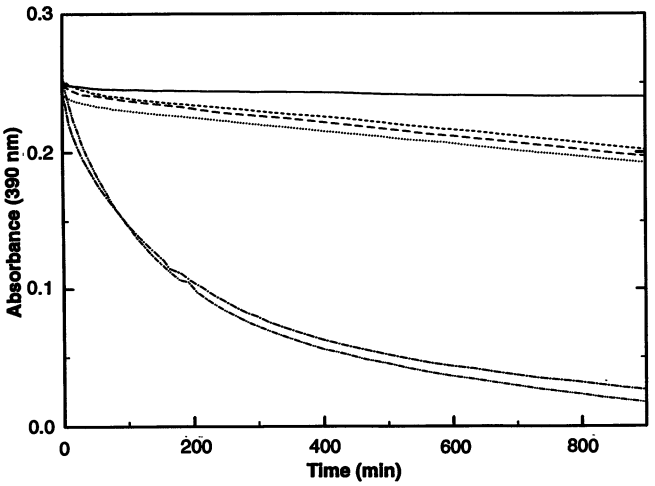

Fig. 2. EDTA chelation of Fe from the Fe:S clusters of Av2-WT and Av2-D125E. Anaerobic quartz cuvettes contained 50 mM tris, pH 8.0, 15 mM $Na_2S_2O_4$, 18.8 μM Av2-WT or Av2-D125E, 4 mM nucleotide, and 5 mM tetrasodium EDTA. The chelation reactions were followed by the decrease in protein absorbance at 390 nm due to destruction of the Fe:S cluster: (——) Av2-WT without added nucleotide, + ADP, or + ATP; (- - - -) Av2-D125E without added nucleotide; (····) Av2-D125E + GTP; (– –) Av2-D125E + AMP; (·–·–) Av2-D125E + ADP; and (··–··) Av2-D125E + ATP.

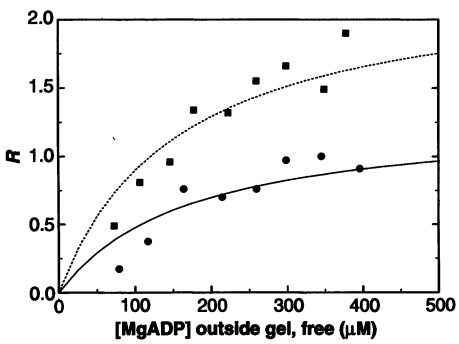

Fig. 3. Binding of MgADP to Av2-WT (●) and Av2-D125E (■). Binding was measured by the gel equilibration method of Hirose and Kano (*18*), as modified by Tso and Burris (*18*). Assays were performed in anaerobic 3.5-ml serum vials. Each vial contained 100 mM NaCl, 25 mM $Na_2S_2O_4$, 0.0 to 600 μM ADP, 35 μM Av2-WT or Av2-D125E, ± 10 mM $MgCl_2$, and 50 mM tris buffer, pH 8.0. Sodium [8-^{14}C]ADP (57.1 mCi/mmol), diluted tenfold with cold ADP to make a 10 mM stock nucleotide solution, was from New England Nuclear. Assays were incubated 30 min at 30°C with shaking, after which 50-μl aliquots of the assay supernatants were removed for liquid scintillation counting. Lines are theoretical fits to the data generated by nonlinear regression analysis with the Marquardt algorithm (*18*) (Table 1); theoretical binding curves are for (——) n = 1.3 and K_D = 170 μM and for (- - -) n = 2.3 and K_D = 155 μM.

Table 1. Summary of thermodynamic constants for the binding of ADP and MgADP to Av2-WT and Av2-D125E by the method of Tso and Burris (*18*). Fitting of the data sets was performed with a nonlinear curve-fitting program based on the Marquardt algorithm (*19*); K_D is the dissociation constant, and n is the number of sites.

Protein	$-Mg^{2+}$		$+ Mg^{2+}$	
	K_D (μM)	n	K_D (μM)	n
Av2-WT	*	*	170 ± 30	1.3
Av2-D125E	185 ± 40	2.0	155 ± 20	2.3

*Under the range of ADP examined (0.0 to 600 μM) less than 0.1 ADP was shown to bind or exchange into Av2-WT.

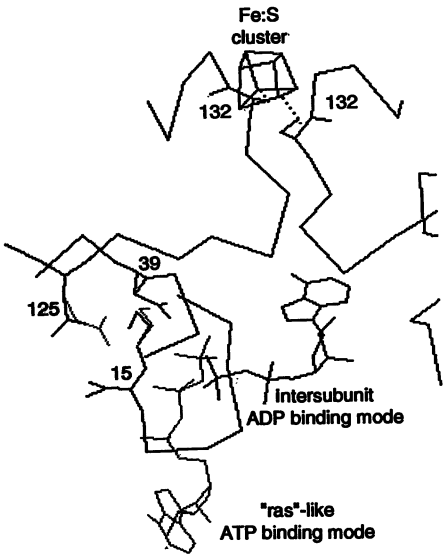

Fig. 4. The nucleotide binding region at interface of the identical Av2-WT subunits. Dark lines represent the Av2 structure showing ADP in the intersubunit binding mode. Lighter lines show the position of ATP in the "Ras-like" binding mode, which is parallel to the subunit interface. Also shown in light lines are the hypothetical positions for Glu^{125} and Lys^{15} as they would be in Av2-D125E.

binding modes in Av2-WT; one, which is partially occupied in the crystal structure, is across the Av2 subunit interface and includes contacts with both subunits; the second site, by analogy to $p21^{ras}$, is parallel to the twofold symmetry axis with contacts predominantly in the individual subunits. In the absence of the triphosphate, Lys^{15} forms a salt bridge with Asp^{125}, or possibly Asp^{39}, or perhaps both. The latter is also positioned to serve as a general base in assisting water attack on the terminal phosphate of ATP (*5*). The potential role of Asp^{125} as the transducer between the nucleotide site and the cluster is clear in Fig. 4. As seen in $p21^{ras}$, movement of the carboxylate toward the nucleotide could lead to a change in the peptide backbone involving the loop between Asp^{125} and Cys^{132} and hence an alteration at the cluster. Substitution by the longer Glu acid side chain would allow the carboxylate to reach the shorter ADP. The loss of Mg requirement in Av2-D125E is more enigmatic although it may be that Lys^{15} binds the terminal phosphates in a way similar to Mg yet is retained in a salt bridge by the longer arm of Glu. At the level of refinement and resolution of the crystal structure, detailed distance measurements are precluded. However, several contacts introduced by the longer Glu side chain would need to be compensated. Among these are interactions with the side chains of Ser^{16} and Ser^{44}, both of which are likely to hydrogen bond to the nucleotide, and most significantly Lys^{15}, which would be displaced toward a salt bridge with Asp^{39}.

Our results should be compared with the recent report by Seefeldt *et al.* (*17*), who found that replacing Lys^{15} by Glu not only abolished MgATP hydrolysis but also the MgATP-dependent chelation. However, they found that nucleotides could still be bound to this altered Av2 (Av2-K15Q), albeit with either lower affinity or in lesser number of sites. A comparison of their result with the crystal structure suggests a possible explanation: perhaps, in the absence of Lys^{15}, the only mode of nucleotide binding is the intersubunit mode. That is, Lys^{15} may be required for the Ras-like binding mode. Furthermore, because chelation does not occur with Av2-K15Q, breaking of the salt bridge between Lys^{15} and Asp^{125}, does not, in and of itself, induce the conformational change.

It is now tempting to speculate about how the two potential modes of nucleotide binding might be related to the electron transfer gating mechanism. From inspection of the model, it appears that the Ras-like orientation would be preferred for an Asp^{39}-assisted attack of water on the ATP γ-phosphate. In this position, Lys^{15} and Asp^{125} would assume the Ras-like interactions with MgATP and the induced conformational change would be transmitted to the cluster. Hydrolysis of ATP would lead to a transition state from which the electron transfer occurs (*10*). Upon release of phosphate, MgADP could move to the intersubunit binding mode with the resulting conformational change closing the gate to prevent electron flow back to the Fe-protein. When Lys^{15} is removed, only the ADP-bound conformation is possible and no nucleotide hydrolysis or cluster movement occurs. In contrast, in Av2-D125E the Ras-like conformation is not only available to the triphosphate but also the diphosphate because of the longer arm of the Glu; the binding for either is transmitted to the cluster. Unlike active Av2-WT, the hydrolysis mechanism is blocked in Av2-D125E, the critical transition to the intersubunit MgADP binding mode does not occur, and the enzyme does not turnover.

We conclude that a general mechanism for nucleotide-dependent signal transduction has evolved. This system includes common features of three-dimensional structure motifs as well as specific amino acid residues. Hence, mechanistic details learned about one system may be relevant to physiologically unrelated systems. For example, from our work on nitrogenase one would expect a D57E alteration in $p21^{ras}$ might change the nucleotide and metal requirements of $p21^{ras}$.

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15. The reported values for nucleotide binding to Fe-protein are quite variable and range from one to two binding sites, with or without cooperativity, and with dissociation constants from <5 μM to >500 mM. Most of these values have been reported for studies on oxidized Fe-protein or Fe-protein from other sources. The variation in values can be attributed to differences in activity of the proteins used, in methods of determination, in estimation of protein concentration and in methods of evaluating the data. Our value of one MgADP site in Av2-WT does not preclude a second weaker site not observed in the nucleotide concentration range used here or, alternatively, a very strong site which does not exchange under our conditions. In either case, Av2-D125E has been altered in the relative affinity of MgADP sites. See G. Yates, in *Biological Nitrogen Fixation*, G. Stacey, R. Burris, H. J. Evans, Eds. (Chapman & Hall, New York, 1992), p. 685, for a recent review of this point.
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20. We thank D. C. Rees for providing the Fe-protein crystal structure coordinates without which much of our speculation would not be possible. J.B.H. is particularly indebted to D. C. Rees for his hospitality and the refreshing, frank discussions during numerous visits to his laboratory. The stimulating conversations with L. Hagerty are also gratefully acknowledged. Supported by the National Science Foundation grant 91-20515.

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Prevention of Protein Denaturation Under Heat Stress by the Chaperonin Hsp60

Jörg Martin, Arthur L. Horwich, F. Ulrich Hartl*

The increased synthesis of heat shock proteins is a ubiquitous physiological response of cells to environmental stress. How these proteins function in protecting cellular structures is not yet understood. The mitochondrial heat shock protein 60 (Hsp60) has now been shown to form complexes with a variety of polypeptides in organelles exposed to heat stress. The Hsp60 was required to prevent the thermal inactivation in vivo of native dihydrofolate reductase (DHFR) imported into mitochondria. In vitro, Hsp60 bound to DHFR in the course of thermal denaturation, preventing its aggregation, and mediated its adenosine triphosphate–dependent refolding at increased temperatures. These results suggest a general mechanism by which heat shock proteins of the Hsp60 family stabilize preexisting proteins under stress conditions.

On exposure to various forms of environmental stress, cells generally respond by increasing the rate of synthesis of a set of highly conserved stress, or heat shock, proteins (Hsps) (*1*). Many stress proteins, including members of the Hsp70 and Hsp60 families, are constitutively expressed and fulfill essential functions as "molecular chaperones" (*2*) under normal cellular conditions. The Hsp60s, which are found in bacterial cytosol as well as in mitochondria and chloroplasts, are high molecular mass, double-ring complexes consisting of 14 ~60-kD subunits (*2*). These "chaperonins" (*3*) interact with early intermediates in the protein folding pathway and mediate the acquisition of the native structure of newly synthesized proteins by releasing the substrate in an adenosine triphosphate (ATP)–dependent process (*4–8*). The ATP-hydrolytic activity of chaperonins is regulated by smaller co-chaperonins, ring complexes of seven ~10-kD subunits (*4*, *9*).

Although most conditions that induce the stress response potentially lead to the accumulation of denatured proteins (*10*), little is known about the mechanisms by which Hsps might function in preventing denaturation or in renaturing damaged proteins (*1*). On exposure of *Escherichia coli* to temperatures between 42° and 46°C, the concentration of the bacterial chaperonin GroEL increases five- to tenfold, reaching up to 12% of total cellular protein. Similarly, the concentration of the mitochondrial Hsp60 of yeast increases two- to threefold at 42°C (*8*, *11*), and that of the co-chaperonin Hsp10 of mammalian mitochondria approximately tenfold under these conditions (*12*). Such induction may reflect an increased requirement for these Hsps in protecting preexisting proteins from denaturation.

A large number of polypeptides were bound to mitochondrial Hsp60 when isolated mitochondria of ^{35}S-labeled *Neurospora crassa* were incubated for 10 min at increased temperatures (Fig. 1). Mitochondrial extracts (*13*) were analyzed by electrophoresis on native polyacrylamide gels, on which Hsp60-bound polypeptides comigrate with the Hsp60 complex. When the bands corresponding to Hsp60 were excised and subjected to electrophoresis on reducing SDS–polyacrylamide gels, a range of polypeptides of 10 to 90 kD that had been associated with Hsp60 was apparent. The amount of bound protein increased significantly after shifting the temperature from 32° to 39° or 46°C (Fig. 1). A small number of proteins comigrated with Hsp60 already at 25°C, an unidentified polypeptide of ~34 kD being most prominent. The total amount of ^{35}S present in the Hsp60-associated polypeptides corresponded to ~10% of that in Hsp60, which is consistent with the observed capacity of the 14-subunit chaperonin to bind only one to two substrate molecules (*4–6*, *14*). The Hsp60-associated proteins did not represent degradation products of Hsp60 (*15*). Protein binding to Hsp60 occurred in the intact mitochondria because it was not prevented when a large excess of α_{s1}-casein was present during preparation of the extracts (Fig. 1); casein binds to the chaperonin despite its stability in solution (*4*). Similar results were obtained with *Saccharomyces cerevisiae* mitochondria (*16*).

Apparently, Hsp60 recognizes a variety of preexisting mitochondrial proteins as

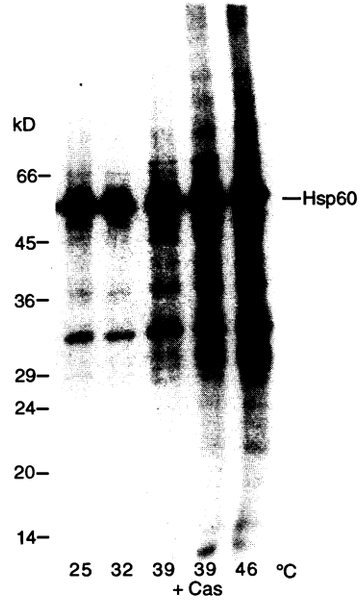

Fig. 1. Temperature-dependent association of proteins with Hsp60 in organello. Aliquots (50 μl) of *Neurospora crassa* mitochondria (5 mg of protein per milliliter) isolated from cells grown in the presence of $^{35}SO_4$ (*28*) were incubated for 10 min at the indicated temperatures in buffer A [0.6 M sorbitol, 20 mM MOPS (pH 7.2), 1 mM dithiothreitol (DTT), and 25 mM KCl] containing 4 mM magnesium acetate, 1 mM ATP, and 2 mM reduced nicotinamide adenine dinucleotide (NADH). Mitochondria were lysed in buffer A containing 7.5 mM cyclohexane diamine tetraacetic acid (to prevent ATP hydrolysis by Hsp60) and 0.075% of the nonionic detergent Genapol (Hoechst, Frankfurt, Germany). One sample, incubated at 39°C, received 50 μg of α_{s1}-casein (Cas) before lysis. Membranes and insoluble material were removed by centrifugation for 15 min at 30,000*g*. The amounts of total ^{35}S-labeled protein in pellet fractions (containing mostly membrane protein) increased from ~30 to 40% after a temperature shift from 25 to 46°C. Approximately 95% of total Hsp60 remained soluble upon incubation of mitochondria at the various temperatures. Supernatant fractions were analyzed by nondenaturing polyacrylamide gel electrophoresis (native PAGE) (*7*). The Hsp60-containing band was excised and analyzed by SDS-PAGE and fluorography. The position of Hsp60 is indicated on the right, and the positions of molecular mass standards (in kilodaltons) are indicated on the left.

J. Martin and F. U. Hartl, Program of Cellular Biochemistry and Biophysics, Rockefeller Research Laboratories, Sloan-Kettering Institute, New York, NY 10021.
A. L. Horwich, Howard Hughes Medical Institute and Department of Human Genetics, Yale School of Medicine, New Haven, CT 06510.
*To whom correspondence should be addressed.