Phenotypic Basis of Reproductive Success in a Social Insect: Genetic and Social Determinants

Laurent Keller* and Kenneth G. Ross

Social insects live in societies that include both reproductive and nonreproductive adults. Understanding the factors that determine which individuals become successful reproductives is necessary to explain the evolution of these societies. The phenotypic effects of the gene *Pgm-3* (or a closely linked gene) that may cause workers of the fire ant *Solenopsis invicta* to selectively execute all queens of a specific genotype were investigated. These effects, which involve differences in queen reproductive development, are expressed only in colonies exhibiting a particular type of social organization (multiple-queen colonies), and it is only in such colonies that selective execution on the basis of genotype occurs. This is an unusual example of genotype-environment interaction in gene expression in which the environmental component is the social environment. The queens executed are, surprisingly, those with the greatest reproductive development. Thus, there is a counterintuitive relation between the potential and realized reproductive success of queens in multiple-queen societies of this ant.

The existence of sterile individuals in social insect colonies poses an evolutionary paradox that can be traced to Darwin, that is, the difficulty of perpetuating sterility via natural selection when bearers of this trait leave no offspring (*1*). A central goal in resolving this paradox has been to understand the factors that determine which adults in a colony become successful reproductives. The identity of such factors, the extent to which their expression is genetically determined, and the mechanisms by which they influence reproductive success are poorly understood in most social insects (*2*, *3*). In the red imported fire ant, *Solenopsis invicta*, a gene (or genes) closely linked to the enzyme-encoding locus *Pgm-3*, or this locus itself, is involved in determining the reproductive roles of queens in multiple-queen (polygynous) colonies; queens of the homozygous genotype *Pgm-3^a/-3^a* never become egg layers in such colonies (*4*). In contrast, *Pgm-3^a/-3^a* is the most common genotype of the single reproductive queens that inhabit each colony of the monogynous social form of this species (*4*, *5*). These findings prompted us to investigate the genetic determinants of reproductive success in this social insect by addressing the following issues: (i) the means by which polygynous queens of the genotype *Pgm-3^a/-3^a* are precluded from becoming functional reproductives (through differential mortality or through preferential entry of the alternate genotypes into the reproductive pool); (ii) the nature of phenotypic differences among queens of different *Pgm-3* genotypes that may be important in the process of differential mortality or entry; and (iii) the role of the social environment in the expression of any such phenotypic differences.

To find out how polygynous queens of the genotype *Pgm-3^a/-3^a* are precluded from becoming reproductives, we designed two experiments that mimicked the processes of recruitment of new reproductive queens in polygynous nests. Queens are adopted after having participated in mating flights (*6*, *7*) or they may mate and start reproducing in their parent nests without having flown if the reproductive queens already present in the colony are lost or die (*7*, *8*). In either circumstance, young queens initiate reproduction in response to a deficit of the pheromones produced by reproductive queens that normally suppress reproductive development of their daughters (*9*, *10*). Workers subsequently readjust queen number by accepting some of these incipient reproductive queens and destroying the remainder (*11*).

In our first experiment, which mimicked recruitment of new queens from within the nest, survival of incipient reproductive queens of the genotype *Pgm-3^a/-3^a* was negligible as compared with the survival of queens of the other two genotypes (Table 1, experiment 1). In our second experiment, which mimicked adoption of queens after a mating flight, not a single incipient reproductive queen of the genotype *Pgm-3^a/-3^a* survived reintroduction into its parent nest (Table 1, experiment 2). Workers in the latter experiment were observed attacking queens within hours after their reintroduction. These results suggest that the genotype *Pgm-3^a/-3^a* is absent among reproductive queens in natural polygynous nests of *S. invicta* because young queens with this genotype are selectively executed by workers as they initiate reproduction.

Selective worker destruction of *Pgm-3^a/-3^a* queens must be the result of phenotypic differences among queens that correlate with their *Pgm-3* genotype and provide workers with cues to discriminate among queens. Worker discrimination among queens is known to be based on pheromonal cues in fire ants (*11*, *12*), and production of these queen pheromones is associated with an individual's reproductive competence (*12*, *13*). Thus, we hypothesized that polygynous queens of different *Pgm-3* genotypes may exhibit different states of reproductive development. Several lines of evi-

Table 1. Survival of incipient reproductive queens of different *Pgm-3* genotypes in two experiments designed to mimic the natural processes of queen recruitment in polygynous *S. invicta* (*26*). Values are frequencies of the three genotypes at the beginning and conclusion of the two experiments, with the relative genotypic proportions shown in parentheses. Experiment 1 mimicked the process of recruitment of new reproductive queens from within a colony after a drop in the number of older reproductives (*27*). Experiment 2 mimicked the process of recruitment of new reproductive queens returning from a mating flight (*28*).

	Pgm-3 genotype		
	aa	*ab*	*bb*
Initial frequencies (*29*)	41 (0.11)	251 (0.67)	84 (0.22)
Experiment 1*	6 (0.02)	239 (0.72)	86 (0.26)
Experiment 2†	0 (0)	208 (0.81)	48 (0.19)

*Of the 378 queens maintained in 19 colony fragments from which the original reproductive queens had been removed, 331 (88%) survived for 10 days. Only six of the survivors had the genotype *Pgm-3^a/-3^a*, yet 36 such individuals were expected if survivorship was distributed randomly across the genotypes. Frequencies of the three genotypes differed significantly between the beginning and end of the experiment ($X_2^2 = 23.6$; $P < 0.001$), but the ratio of *Pgm-3^a/-3^b* to *Pgm-3^b/-3^b* genotypes did not change over this period ($X_1^2 = 0.17$; $P > 0.6$). Thus, survivorship was distributed randomly across the latter two genotypes, whereas *Pgm-3^a/-3^a* queens had a significantly diminished survival probability. †Of the 378 isolated queens that were reintroduced into their 19 parent colonies, 256 (68%) survived for 1 week. None of these possessed the genotype *Pgm-3^a/-3^a* (28 such individuals were expected if survivorship was random). Frequencies of the three genotypes at the beginning of the experiment differed significantly from those at the end ($X_2^2 = 33.3$; $P < 0.001$), but the ratio of *Pgm-3^a/-3^b* to *Pgm-3^b/-3^b* genotypes did not change significantly during this same period ($X_1^2 = 3.36$; $P > 0.05$), indicating that only *Pgm-3^a/-3^a* queens had a significantly lower survivorship than expected if mortality was distributed randomly across the genotypes (*30*).

L. Keller, Museum of Comparative Zoology, Harvard University, Cambridge, MA 02138.
K. G. Ross, Department of Entomology, University of Georgia, Athens, GA 30602.

*Present address: Zoologisches Institut, Universität Bern, Ethologische Station Hasli, Wohlenstrasse 50a, CH-3032 Interkappelen, and Institut de Zoologie et d'Ecologie Animale, Université de Lausanne, Bâtiment de Biologie, CH-1015 Lausanne, Switzerland.

dence support this hypothesis. Winged (nonreproductive) queens of the genotype *Pgm-3*^{a}/-3^a are heavier than other winged queens (Table 2), a difference that is accentuated once these queens are free to initiate oogenesis after being removed from pheromonal inhibition by their reproductive nestmates (Fig. 1). Comparison of the weights of winged queens from a single polygynous colony (n = 87) revealed that the weight differences are attributable to differences in abdomen weight (F = 7.04; df = 2, 84; $P < 0.001$) rather than thorax weight (F = 2.82; df = 2, 84; $P > 0.05$), with the only significant differences in abdomen weight occurring between *Pgm-3*^{a}/-3^a queens and the others (Scheffe's test; α = 0.05). Accumulation of fat and glycogen reserves in the abdomen is a characteristic feature of reproductive development in ant queens (*14*), suggesting that the abdominal weight differences observed in fire ant queens of different *Pgm-3* genotypes reflect differences in the reproductive state of these individuals.

Direct evidence for this conclusion comes from studies of wing-shedding behavior (which is linked to the onset of oogenesis) (9) and egg laying. Winged polygynous queens of the genotype *Pgm-3*^{a}/-3^a shed their wings significantly more often and lay significantly more eggs than queens of the other genotypes after being removed from their parent nests (Table 2). Thus, *Pgm-3*^{a}/-3^a queens seem not only to be in a relatively advanced state of reproductive development while in the nest, but they also begin reproducing at an accelerated pace, as compared with other queens, when they are removed from the inhibitory influence of reproductive queens. The tight coupling of a queen's reproductive state and pheromone production (*12*, *13*) suggests that the reproductively precocious *Pgm-3*^{a}/-3^a queens produce elevated amounts of queen pheromone and that the workers use the amount of pheromone produced as a cue to recognize and selectively execute these queens.

The equivalence of *Pgm-3*^{a}/-3^b and *Pgm-3*^{b}/-3^b queens in terms of weight and pace of oogenesis (Table 2 and Fig. 1) suggests that *Pgm-3*^{a} acts as a recessive allele with respect to effects on these traits, and perhaps with respect to queen pheromone production as well (*15*). The observation that the genotype *Pgm-3*^{a}/-3^a is completely absent among reproductive queens in natural polygynous nests, whereas the other two genotypes are represented in the same relative proportions in both reproductive and nonreproductive queens (*4*), can be explained by the workers' failure to distinguish between the reproductive or pheromonal phenotypes (or both) of *Pgm-3*^{a}/-3^b and *Pgm-3*^{b}/-3^b queens.

Although *Pgm-3*^{a}/-3^a is absent among reproductive queens of the polygynous social form of *S. invicta*, it is the most common genotype found among reproductive queens of the monogynous social form of this species (*4*). Because the phenotypic correlates of *Pgm-3*^{a}/-3^a discovered in the polygynous form lead to the certain death of queens with this genotype, the question arises as to whether *Pgm-3* genotype is associated with similar phenotypic differences among monogynous queens. No statistically significant weight differences were found among winged monogynous queens of different *Pgm-3* genotypes when they

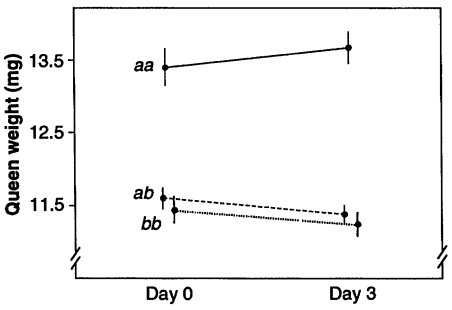

Fig. 1. Weights of winged polygynous queens (mean ± SE) of different *Pgm-3* genotypes when taken directly from their parent colonies (day 0) and after 3 days of isolation from reproductive nestmates (day 3) (*24*). Queens of the genotype *Pgm-3*^{a}/-3^a gained weight significantly more often than queens of the other two genotypes (X_1^2 = 7.84; $P < 0.01$), whereas there was no statistically significant difference between *Pgm-3*^{a}/-3^b and *Pgm-3*^{b}/-3^b queens (X_1^2 = 0.05; $P > 0.75$). Genotype at *Pgm-3* had a significant effect on weight at day 0 (F = 25.5; df = 2, 370; $P < 0.001$) as well as at day 3 (F = 35.1; df = 2, 370; $P < 0.001$). The genotypic effect at both times is attributable to the *Pgm-3*^{a}/-3^a queens being heavier than the others (Scheffe's tests; $P < 0.001$ for both days); *Pgm-3*^{a}/-3^b and *Pgm-3*^{b}/-3^b queens were indistinguishable in weight on both days (Scheffe's tests; $P > 0.5$ for both days) (*25*). For *aa*, n = 41; for *ab*, n = 249; for *bb*, n = 83.

Table 2. Weight and reproductive development of winged polygynous queens of different *Pgm-3* genotypes. Values for weight and number of eggs laid are means ± SE. Sample sizes (numbers of individuals) are shown in parentheses for each genotype (*31*).

	Pgm-3 genotype		
	aa	*ab*	*bb*
Weight (milligrams)*	12.7 ± 0.18 (n = 71)	11.2 ± 0.06 (n = 469)	11.2 ± 0.09 (n = 141)
Proportion with shed wings†	0.73 (n = 71)	0.13 (n = 469)	0.14 (n = 141)
Number of eggs laid in five hours‡	2.10 ± 0.49 (n = 41)	0.19 ± 0.05 (n = 249)	0.06 ± 0.04 (n = 83)

*Two-way analyses of variance showed that the weights of queens were significantly influenced by their *Pgm-3* genotype (F = 40.0; df = 2, 601; $P < 0.001$) and colony of origin (F = 9.72; df = 31, 601; $P < 0.001$). Pairwise comparisons revealed that *Pgm-3*^{a}/-3^a queens were heavier than queens of the other two genotypes (Scheffe's test; $P < 0.001$), whereas the weights of *Pgm-3*^{a}/-3^b and *Pgm-3*^{b}/-3^b queens were statistically indistinguishable (Scheffe's test; $P > 0.99$) (*32*). †Proportion of queens shedding their wings varied significantly by genotype (X_2^2 = 149; $P < 0.001$), with no statistically significant difference between *Pgm-3*^{a}/-3^b and *Pgm-3*^{b}/-3^b queens (X_1^2 = 0.25; $P > 0.6$) (*33*). ‡Number of eggs laid varied significantly by genotype (F = 19.8; df = 2, 322; $P < 0.001$) and colony of origin (F = 2.89; df = 18, 322; $P < 0.001$), with the only significant pairwise genotypic differences occurring between *Pgm-3*^{a}/-3^a and the other two genotypes (Scheffe's test; $P < 0.001$) (*34*).

Table 3. Weight of monogynous queens of different *Pgm-3* genotypes. Values in the genotype columns are mean weights ± SE (in milligrams) of nonreproductive queens collected directly from their parent colonies (nests 1 to 4) or immediately after a mating flight. Sample sizes (numbers of individuals) are shown in parentheses for each genotype. Values of t for comparisons between two genotypic classes, or of F for the comparison among all three classes, are presented along with their associated probability values (P) (*35*).

	Pgm-3 genotype			t (F)	P
	aa	*ab*	*bb*		
Nest 1	13.6 ± 0.23 (n = 26)	13.9 ± 0.13 (n = 33)	—	1.18	>0.24
Nest 2	13.8 ± 0.19 (n = 29)	13.5 ± 0.15 (n = 31)	—	1.10	>0.27
Nest 3	11.8 ± 0.28 (n = 20)	11.4 ± 0.23 (n = 25)	—	0.88	>0.38
Nest 4	13.6 ± 0.18 (n = 27)	14.0 ± 0.16 (n = 18)	—	1.65	>0.10
Mating flight	15.1 ± 0.15 (n = 47)	14.9 ± 0.17 (n = 51)	15.9 ± 0.45 (n = 5)	1.74	>0.18

were collected inside their nests or immediately after a mating flight (Table 3). Furthermore, similar proportions of winged *Pgm-3^a/-3^a* and *Pgm-3^a/-3^b* queens from a single monogynous colony shed their wings in the laboratory within 24 hours after their mother queen was removed (0.41 and 0.59; $n = 90$; $\chi_1^2 = 2.85$; $P > 0.1$). Thus, the effects of *Pgm-3* genotype on queen reproductive phenotype that we observed are expressed only in the social environment characteristic of polygynous nests (*15*). This differential expression may result from differences in the cumulative levels of queen pheromone present in individual nests of the alternate social forms (*11, 12*) because these different levels also appear to affect worker phenotypes in this species (*16*).

This study shows that the genotype *Pgm-3^a/-3^a* is never found among reproductive queens of the polygynous form of *S. invicta* because queens bearing this genotype are killed by workers as they initiate oogenesis. Furthermore, workers apparently recognize variation in reproductive or pheromonal phenotype (or both) that correlates with the *Pgm-3* genotype, and they selectively execute *Pgm-3^a/-3^a* queens by using this variation as the basis for discrimination. Finally, the lack of observable phenotypic differences among queens of different *Pgm-3* genotypes in the monogynous form suggests that the effects of this gene on reproductive competence are dependent on social environment, that is, genotype-environment interactions are important in the phenotypic expression of the gene. It is surprising that polygynous workers invariably destroy queens of the greatest reproductive potential because fitness of social insect queens generally is expected to be positively correlated with their fecundity (*2, 3, 17*). If polygyny is advantageous for fire ants under some ecological conditions, as has been suggested for other social insects (*3, 18*), then worker execution of reproductively superior queens may represent a mechanism selected to maintain numerous queens within a colony. Colony queen number is inversely related to individual fecundity in fire ants and other social insects (*19*), so that worker execution of *Pgm-3^a/-3^a* queens presumably prevents these most fecund of individuals from becoming the dominant reproductives in a nest and driving the population toward reduced colony queen numbers or even monogyny.

REFERENCES AND NOTES

1. C. R. Darwin, *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life* (John Murray, London, 1859); J. Seger, in *Behavioural Ecology: An Evolutionary Approach*, J. R. Krebs and N. B. Davies, Eds. (Blackwell, Cambridge, United Kingdom, ed. 3, 1991), pp. 338–373.
2. E. O. Wilson, *The Insect Societies* (Belknap, Cambridge, MA, 1971); C. D. Michener, *The Social Behavior of Bees* (Belknap, Cambridge, MA, 1974); W. Engels, Ed., *Social Insects: An Evolutionary Approach to Castes and Reproduction* (Springer-Verlag, Berlin, 1990); R. Gadagkar, S. Bhagavan, K. Chandrashekara, C. Vinutha, *Ecol. Entomol.* **16**, 435 (1991); P.-F. Röseler, in *The Social Biology of Wasps*, K. G. Ross and R. W. Matthews, Eds. (Cornell Univ. Press, Ithaca, NY, 1991), pp. 309–335.
3. B. Hölldobler and E. O. Wilson, *The Ants* (Belknap, Cambridge, MA, 1990).
4. K. G. Ross, *Nature* **355**, 347 (1992).
5. The allele *Pgm-3^a* is maintained in the polygynous form in northern Georgia by immigration of males from the neighboring monogynous population, where this allele is common. This gene flow is inferred from three lines of evidence [see (*4*); K. G. Ross and D. D. Shoemaker, *Evolution*, in press]: (i) excess heterozygosity exists at *Pgm-3* in nonreproductive females of the polygynous but not the monogynous form, the pattern expected if polygynous queens mate predominantly or solely with monogynous males and given the strong *Pgm-3* allele frequency differences that exist between sexuals of the two forms; (ii) reconstruction of *Pgm-3* mating types by progeny analyses indicates that queens of the polygynous form mate predominantly with males bearing the allele that is most common in the monogynous form, *Pgm-3^a*; and (iii) allele frequencies at 12 additional protein-encoding loci and numbers of sex-determination alleles are similar between the two forms.
6. B. M. Glancey and C. S. Lofgren, *Fla. Entomol.* **71**, 581 (1988).
7. S. D. Porter, *J. Entomol. Sci.* **26**, 474 (1991).
8. E. L. Vargo and S. D. Porter, *Ann. Entomol. Soc. Am.* **82**, 307 (1989).
9. D. J. C. Fletcher and M. S. Blum, *Science* **212**, 73 (1981).
10. ———, *J. Comp. Physiol. A* **153**, 467 (1983).
11. D. J. C. Fletcher, in *Advances in Invertebrate Reproduction*, M. Porchet, J.-C. Andries, A. Dhainaut, Eds. (Elsevier, Amsterdam, 1986), vol. 4, pp. 305–316.
12. D. J. C. Fletcher and M. S. Blum, *Science* **219**, 312 (1983).
13. D. E. Willer and D. J. C. Fletcher, *Physiol. Entomol.* **11**, 475 (1986).
14. L. Keller and L. Passera, *Oecologia* **80**, 236 (1989); L. Passera and L. Keller, *J. Comp. Physiol. B* **160**, 207 (1990).
15. A gene (or genes) closely linked to *Pgm-3* actually may be responsible for the phenotypic effects we describe.
16. Workers of the polygynous form of *S. invicta* are consistently smaller than workers of the monogynous form, presumably because the high cumulative levels of queen pheromone in polygynous colonies relative to monogynous colonies inhibit the growth and development of worker brood [L. Greenberg, D. J. C. Fletcher, S. B. Vinson, *J. Kans. Entomol. Soc.* **58**, 9 (1985)].
17. M. J. West-Eberhard, *Misc. Publ. Museum Zool. Univ. Mich.* **140**, 1 (1969); K. G. Ross, *Behav. Ecol. Sociobiol.* **23**, 341 (1988); L. Keller, *Oikos*, in press.
18. M. V. Brian, *Social Insects: Ecology and Behavioural Biology* (Chapman & Hall, London, 1983); P. Nonacs, *Evolution* **42**, 566 (1988); L. Keller, Ed., *Queen Number and Sociality in Insects* (Oxford Univ. Press, Oxford, in press).
19. E. L. Vargo and D. J. C. Fletcher, *Physiol. Entomol.* **14**, 223 (1989); L. Keller and E. L. Vargo, in *Queen Number and Sociality in Insects*, L. Keller, Ed. (Oxford Univ. Press, Oxford, in press).
20. D. P. Jouvenaz, G. E. Allen, W. A. Banks, D. P. Wojcik, *Fla. Entomol.* **60**, 275 (1977).
21. L. Morel, R. K. Vander Meer, C. S. Lofgren, *Ann. Entomol. Soc. Am.* **83**, 642 (1990).
22. K. G. Ross and D. J. C. Fletcher, *Behav. Ecol. Sociobiol.* **17**, 349 (1985).
23. K. G. Ross, *Am. Nat.*, in press.
24. These data were collected from the same individuals that were isolated in colony fragments and subjected to egg-laying tests (see Table 2).
25. Weight differences between queens in this experiment are important at the colony level as well as population level. Median weights of *Pgm-3^a/-3^a* queens were greater than the median weights of nestmate queens of the other genotypes in 10 of the 13 colonies containing *Pgm-3^a/-3^a* queens at day 0 (sign test; $P < 0.05$), whereas all 41 *Pgm-3^a/-3^a* queens were heavier than the median weight of their other nestmates at day 3 (sign test comparing median weights of *Pgm-3^a/-3^a* queens and other nestmate queens; $P < 0.001$).
26. The queens originated from 19 polygynous colonies collected in northern Georgia in the spring of 1991. Entire colonies were transferred into laboratory rearing units with the use of standard procedures (*20*), and then the number of reproductive queens in each colony was reduced to four (the minimum number found in any of the 19 colonies).
27. A fragment of each of the source colonies containing several thousand worker brood and adults and 20 winged nonreproductive queens, but no reproductive queens, was maintained in the laboratory for 10 days. All surviving queens were then collected from these fragments, and their *Pgm-3* genotypes were determined with the use of electrophoresis (*4*).
28. Twenty small fragments of each source colony containing several hundred worker brood and adults and a winged nonreproductive queen were maintained in the laboratory for 3 days, after which these isolated queens were returned to their parent colonies. [Queens were reintroduced into the colonies from which they originated in order to eliminate nestmate discrimination as a factor influencing rates of acceptance (*11, 21*)]. All queens that survived for 1 week after reintroduction were collected and genotyped.
29. We estimated initial frequencies of *Pgm-3* genotypes among winged queens in the source colonies by collecting and genotyping 20 such queens from each colony when the two experiments were started. Because genetic variation is distributed relatively evenly across polygynous nests [that is, nestmate relatedness is low (*22, 23*)], most of the source colonies were expected to have at least some winged queens of the genotype *Pgm-3^a/-3^a*. The presence of such queens was confirmed in 14 of the 19 source colonies from the initial samples of only 20 individuals per colony.
30. The comparatively poor survivorship of *Pgm-3^a/-3^a* queens revealed for the population of colonies in both recruitment experiments also is evident within the individual colonies. The proportion of *Pgm-3^a/-3^a* queens from individual colonies at the conclusion of each experiment invariably was equal to or less than the initial proportion of such queens in the same colonies, an outcome that departs significantly from the null expectation of equally frequent increases or decreases in the proportions (sign test; $P < 0.001$ for both experiments).
31. The queens originated from 32 polygynous colonies (17 to 38 queens per colony) that were collected and reared in the laboratory under standard conditions (*20, 22*). Weights were determined for mature winged (nonreproductive) queens immediately after they were removed from their parent colonies. The proportion of such queens shedding their wings was determined after the queens were individually isolated in small fragments of their parent colonies containing several hundred worker brood and adults for a period of 3 days. The number of eggs laid was determined at this same time for a subset of the isolated queens from 19 of the parent colonies (19 to 20 queens per colony) with the use of a standardized 5-hour egg-laying test developed for fire ant queens (*10*).
32. A significant effect of the interaction between genotype and colony of origin on queen weight ($F = 2.11$; df = 46; $P < 0.001$) suggests that the genotypic influence on weight varies among the study colonies in this experiment. Nonetheless, the median weight of *Pgm-3^a/-3^a* queens was greater than the median weight of nestmate

queens of the other genotypes in 15 of the 18 colonies containing *Pgm-3^a/-3^a* queens, a pattern that departs significantly from the null expectation of equal frequencies of higher and lower median weights for *Pgm-3^a/-3^a* queens (sign test; $P < 0.005$). Thus, weight differences among the genotypes are important at the colony level as well as at the population level.

33. At the colony level, the proportion of *Pgm-3^a/-3^a* queens shedding their wings invariably was greater than the proportion of nestmate queens of the other genotypes that shed their wings in the 15 colonies containing two or more *Pgm-3^a/-3^a* queens (sign test; $P < 0.001$).
34. Colony of origin and *Pgm-3* genotype interacted significantly to influence the number of eggs laid ($F = 2.68$; df = 30; $P < 0.001$). However, *Pgm-3^a/-3^a* queens invariably laid as many or more eggs than the median number laid by nestmate queens of the alternate genotypes (sign test comparing median numbers of eggs laid by *Pgm-3^a/-3^a* queens and other nestmate queens; $P < 0.005$), showing that the effect of genotype on fecundity is important in individual colonies as well as the entire population. All statistical analyses on egg counts were done on values transformed as $(x + 0.5)^{1/2}$ [R. R. Sokal and F. J. Rohlf, *Biometry: The Principles and Practice of Statistics in Biological Research* (Freeman, San Francisco, ed. 2, 1981)].
35. Nests 1 to 4 were collected from a monogynous population in northern Georgia in the spring of 1991 and reared for 10 days in the laboratory. These four nests were selected on the basis of having the genotype *Pgm-3^a/-3^a* segregating with *Pgm-3^a/-3^b* among winged daughter queens. The single reproductive queens in monogynous nests mate with a single haploid male (*22*), so that alternate homozygotes at single loci are never present in the same nest (this explains why the genotype *Pgm-3^b/-3^b* is not represented in these four nests). Queens from the mating flight were collected in the summer of 1991 from the same monogynous population as nests 1 to 4 (the polygynous form does not occur in this area). Mating flights of *S. invicta* are synchronized over large areas, and sexuals are capable of flying considerable distances during these flights [G. P. Markin, J. H. Dillier, S. O. Hill, M. S. Blum, H. R. Hermann, *J. Ga. Entomol. Soc.* **6**, 145 (1971)], with the result that these queens are likely to have originated from many different nests (this explains why all three genotypes are represented in this sample).
36. Funded in part by the Swiss National Science Foundation (grant 823A-0283650), the Janggen-Pöhn Stiftung, the National Geographic Society, and the Georgia Agricultural Experiment Stations of the University of Georgia. We thank J. J. Boomsma, D. Haig, N. E. Pierce, H. K. Reeve, and E. O. Wilson for comments on the manuscript.

5 October 1992; accepted 4 February 1993

Kinetics of Folding of the All–β Sheet Protein Interleukin-1β

Paul Varley,* Angela M. Gronenborn,† Henriette Christensen, Paul T. Wingfield, Roger H. Pain,‡ G. Marius Clore†

The folding of the all–β sheet protein, interleukin-1β, was studied with nuclear magnetic resonance (NMR) spectroscopy, circular dichroism, and fluorescence. Ninety percent of the β structure present in the native protein, as monitored by far-ultraviolet circular dichroism, was attained within 25 milliseconds, correlating with the first kinetic phase determined by tryptophan and 1-anilinonaphthalene-8-sulfonate fluorescence. In contrast, formation of stable native secondary structure, as measured by quenched-flow deuterium-hydrogen exchange experiments, began after only 1 second. Results from the NMR experiments indicated the formation of at least two intermediates with half-lives of 0.7 to 1.5 and 15 to 25 seconds. The final stabilization of the secondary structure, however, occurs on a time scale much greater than 25 seconds. These results differ from previous results on mixed α helix–β sheet proteins in which both the α helices and β sheets were stabilized very rapidly (less than 10 to 20 milliseconds).

It has been observed that helix formation can occur within the low millisecond time scale when unfolded proteins are transferred into refolding conditions (*1*). This was perhaps not surprising because the hydrogen bonding interactions involved in helix formation are local, extending over only four residues for one turn of helix. Whereas both α helices and β sheets form secondary structure elements within a protein, hydrogen bonding in β sheet formation involves interactions between distant parts of the polypeptide chain. Hence, it was less expected when quenched-flow deuterium-hydrogen (D-H) exchange protection experiments on mixed α helix–β sheet proteins showed that the kinetics of formation of stable β sheet are similar to those of helices (*2*). Theoretical studies have suggested that only when there is significant stabilization of the β structure will its rate of formation be compatible with the rates of folding of the proteins referred to above (*3*). Whether this rapid stabilization is adequately accounted for by hydrophobic collapse and compaction (*4*) or whether other more specific interactions are required is an important question in protein folding.

To further elucidate the mechanisms of β structure formation during the folding process, we studied the folding of the all–β protein interleukin-1β (IL-1β) (*5*) with techniques sensitive to the conformation, stability, and environment of the secondary structure elements. Interleukin-1β was chosen as a model system for three reasons: (i) complete ^{1}H, ^{15}N, and ^{13}C resonance assignments have been obtained (*6*); (ii) the high-resolution three-dimensional structure has been determined by NMR spectroscopy and x-ray crystallography (*7–9*); and (iii) the equilibria and conditions of its reversible unfolding have been established (*10*). Whereas equilibrium unfolding is two-state, the protein refolds kinetically through a significantly populated, compact intermediate or molten globule that appears to exhibit 80 to 90% of the secondary structure content present in the native protein, as monitored by far-ultraviolet circular dichroism (far-UV CD) ellipticity, but little or no stable tertiary structure (*11*).

The kinetics of β structure formation were monitored by stopped-flow far-UV CD (*12*). At an absorbance of 225 nm, the unfolded and native proteins exhibited mean ellipticities of −3000 and −1200 deg cm^2 dmol^{-1}, respectively, so that the ellipticity becomes less negative as the protein refolds from the unfolded to the native conformation (*10*). On refolding at 4°C, the earliest observed value of ellipticity (after a mixing artifact) was about −1400 deg cm^2 dmol^{-1}, showing that ~90% of the ellipticity was regained within 25 ms in a process with a half-life ($t_{1/2}$) that was less than the dead time (10 ms) of the stopped flow apparatus. Subsequently, there was a slow phase of low amplitude (~10%) with a $t_{1/2} \sim 6$ s.

The rapid formation of β structure as monitored by CD was paralleled by the fluorescence of the single tryptophan residue (Fig. 1A) and bound 1-anilinonaphthalene-8-sulfonate (ANS) (Fig. 1B) (*13*). In the native structure, the fluorescence spectrum of Trp^{120} has a maximum emission wavelength of 344.5 nm (*10*), characteristic of its partially buried state (*7*). Analysis of the tryptophan and ANS fluorescence data suggested a folding pathway with formation of three intermediates

P. Varley, Laboratory of Chemical Physics, Building 2, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (NIH), Bethesda, MD 20892, and Department of Biochemistry and Genetics, University of Newcastle-upon-Tyne, Newcastle NE2 4HH, United Kingdom.
A. M. Gronenborn and G. M. Clore, Laboratory of Chemical Physics, Building 5, Room 132, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD 20892.
H. Christensen and R. H. Pain, Department of Biochemistry and Genetics, University of Newcastle-upon-Tyne, Newcastle NE2 4HH, United Kingdom.
P. T. Wingfield, Protein Expression Laboratory, Building 6B, Office of the Director, NIH, Bethesda, MD 20892.

*Present address: National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, United Kingdom.
†To whom correspondence should be addressed.
‡Present address: Unité de Biochimie Cellulaire, Institut Pasteur, 28 Rue du Dr. Roux, 75724 Paris Cedex 15, France.