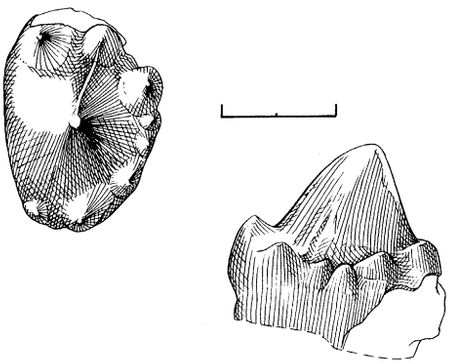

Fig. 4. Occlusal (left) and labial (right) views of the posteriormost right mandibular "cheek" tooth of CD-1-1. Anterior is to the top in the left figure, to the right in the right figure. Scale bar, 2 mm.

Young Nile crocodiles have a diet consisting mainly of insects, and as they grow larger they eat proportionately more vertebrates and fewer insects (*15*). If the Malawi crocodylian had a comparable life history, then the small, subadult fossil specimens were primarily insectivorous. However, the pronounced differences between the feeding mechanisms of the Malawi crocodylian and living crocodylians suggest a different or more specialized diet for the fossil form.

Several amniote groups have independently evolved the capacity for fore-aft movement of the mandible during adduction (*16*). Tortoises, and to a lesser extent some other turtles, retract their mandible in a palinal (front-to-back) motion (*17*), and extinct dicynodont (*18*) and tritylodontid (*19*) therapsids and multituberculate mammals (*11*) are inferred to have had palinal movement. Proal movement is, to our knowledge, only found in some mammals, especially rodents (*20*), and in sphenodontid rhynchocephalians (*21*), including the sole living member, *Sphenodon punctatus* (the tuatara). Anterior movement of the mandible in *Sphenodon* is accomplished by the M. pterygoideus, supporting our interpretation that this muscle was responsible for the same action in the Malawi crocodylian.

All of the amniotes with fore-aft mandibular movement lack other forms of cranial kinesis. This suggests that there is a functional relation between the evolution of fore-aft mandibular movement and having an akinetic skull. The evolution of a mechanism for intraoral food processing in the Malawi crocodylian may also have been related to its possession of a secondary palate, which could have allowed for the maintenance of breathing during food processing, as in living mammals.

REFERENCES AND NOTES

1. R. Steel, *Handbook of Paleoherpetology* (Fischer Verlag, New York, 1973), vol. 16; E. Buffetaut, *Soc. Geol. Fr. Mem. 142* (1982), p. 1; B. Peyer, *Comparative Odontology* (University of Chicago, Chicago, 1968).
2. A. B. Busbey, thesis, University of Chicago, Chicago, IL (1982).
3. F. Dixey, *Trans. R. Soc. S. Afr.* **16**, 55 (1928); J. Kemp, *Malawi Geol. Surv. Dept. Bull.* **41**, 1 (1975).
4. Specimen numbers CD-1-1, CD-1-2, CD-1-3, CD-6-1-1, and CD-6-1-43, and other material, will be housed in the Malawi Department of Antiquities in Lilongwe.
5. J. Clark, thesis, University of Chicago, Chicago, IL (1986); M. J. Benton and J. M. Clark, in *The Phylogeny and Classification of Tetrapods*, M. J. Benton, Ed. (Clarendon Press, Oxford, 1988), vol. 1, p. 295.
6. In a cladistic classification the mesosuchian grade is grouped with the Eusuchia in the Mesoeucrocodylia [K. Whetstone and P. Whybrow, *Occ. Pap. Mus. Nat. Hist. Univ. Kansas 106* (1983)].
7. Z. B. de Gasparini, *Ameghiniana* **8**, 83 (1971). *Notosuchus* is often grouped with several other genera in an Infraorder Notosuchia, but the monophyly of this group is questionable (*5*) and other "notosuchians" lack the three characters shared by *Notosuchus* and the Malawi crocodylian.
8. L. Dollo, *Bull. Cl. Sci. Acad. R. Belgique* **1914**, 288 (1914). *Araripesuchus*, which is known from Brazil, Niger, and Cameroon [E. Buffetaut and P. Taquet *Nature* **280**, 486 (1979); L. L. Jacobs *et al.*, *ibid.* **336**, 158 (1988)], is often placed in the Notosuchidae, but it lacks the derived features of this family (*5*). The Malawi form is therefore the first notosuchid known from Africa and provides further evidence for a Cretaceous fauna endemic to Gondwanaland. J. F. Bonaparte, *Actas IV Cong. Argentino Paleontol. Bioestrat* **2**, 48 (1986); J. F. Bonaparte and Z. Kielan-Jaworowska, *Occ. Pap. Tyrrell Mus. Paleontol.* **3**, 24 (1987).
9. This feature has been independently noted in *Notosuchus* by J. F. Bonaparte (*5*).
10. R. G. Every and W. G. Kühne, *Zool. J. Linn. Soc.* **50** (1971), suppl. 1, p. 23.
11. D. W. Krause, *Paleobiol.* **8**, 265 (1982).
12. F. H. Edgeworth, *The Cranial Muscles of Vertebrates* (Macmillan, London, 1935); G.-H. Schumacher, in *Biology of the Reptilia*, C. Gans and T. Parsons, Eds. (Academic Press, New York, 1973), vol. 4, p. 101.
13. The large size of the M. pterygoideus in living crocodylians is reflected in the size of the flange of the pterygoid bone. The pterygoid flange of the Malawi crocodylian is comparable in size to that of living crocodylians. The supratemporal fenestrae, which are the site of origin for another major mandibular adductor, are very small in the Malawi form, further indicating an emphasis on the M. pterygoideus.
14. K. M. Hiiemae and A. W. Crompton, in *Functional Vertebrate Morphology*, M. Hildebrand *et al.*, Eds. (Belknap, Cambridge, MA, 1985), p. 262.
15. H. B. Cott, *Trans Zool. Soc. London* **29**, 211 (1961).
16. D. M. Bramble and D. B. Wake, in *Functional Vertebrate Morphology*, M. Hildebrand *et al.*, Eds. (Belknap, Cambridge, MA, 1985), p. 230.
17. D. R. Bramble, *Copeia* **1974**, 102 (1974).
18. A. W. Crompton and N. Hotton III, *Postilla* **109**, 1 (1967).
19. A. W. Crompton, *Bull. Brit. Mus. (Nat. Hist.), Geology* **21**, 29 (1972); H.-D. Sues, *Harvard Mus. Comp. Zool. Bull.* **151**, 217 (1986).
20. W. A. Weijs, *J. Morphol.* **145**, 107 (1975).
21. G. C. Gorniak, H. I. Rosenberg, C. Gans, *ibid.* **171**, 321 (1982); G. S. Throckmorton, J. A. Hopson, P. Parks, *J. Paleontol.* **55**, 586 (1981).

22. The fieldwork in Malawi was supported by the National Geographic Society and Southern Methodist University. Study of the specimens has been supported by NSF grants BSR 8700539 to L.L.J. and BSR 840118 to J.M.C. We thank our colleagues at the Malawi Department of Antiquities, particularly G. Mgomezulu, Y. M. Juwayeyi, F. Morocco, and M. Chilinda and Z. Kaufulu of the University of Malawi. Thanks also to D. Winkler, M. Hildebrand, B. Shaffer, J. Bonaparte, S. Carlson, and A. Busbey. Figures 2 and 4 are by L. Sadler and Fig. 3 is by L. Meszoly.

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Transfer of a Protein Encoded by a Single Nucleus to Nearby Nuclei in Multinucleated Myotubes

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Specialized regions of muscle fibers may result from differential gene expression within a single fiber. In order to investigate the range of action of individual nuclei in multinucleated myotubes, C2 myoblasts were transfected to obtain stable cell lines that express a reporter protein that is targeted to the nucleus. Hybrid myotubes were then formed containing one or a few transfected nuclei as well as a large number of nuclei from the parental strain. In order to determine how far the products of a single nucleus extend, transfected nuclei were labeled with [^{3}H]thymidine before fusion and the myotubes were stained to identify the reporter protein. In such myotubes the fusion protein was not confined to its nucleus of origin, but was restricted to nearby nuclei.

IN ORDER TO CONSTRUCT AND MAINTAIN specialized subcellular domains, large extended cells like neurons and muscle fibers must be able to distribute proteins differentially within the cell. Muscle fibers are multinucleated; an adult muscle fiber may contain hundreds of nuclei evenly distributed throughout its length, each capable of producing mRNA and protein. Muscle fibers, nevertheless, produce proteins that have a restricted distribution within the fiber. A classic example is the acetylcholine receptor (AChR), which is concentrated at the neuromuscular junction (*1*). Another example is the demonstration by Salviati *et al.* (*2*) that a fast foreign nerve induces the production of fast myosin in dually innervated slow muscle fibers, but only in the region of the ectopic, fast end plate.

Restricted protein distribution in muscle

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cells may reflect differences in gene expression within a single fiber: the AChR mRNA is more abundant in the end plate region of muscle than elsewhere (*3*), and in situ hybridization shows that mRNA for the α subunit of the AChR is associated with nuclei close to the synapse (*4*). Thus activators and repressors involved in the regulation of mRNA may also have a restricted distribution. Such signals may be transferred from one nucleus to another (*5*).

To investigate the possible range of action of individual nuclei within multinucleated muscle cells, we have generated stable transfectants of the C2 mouse muscle cell line that express a reporter protein fused to nuclear localization signals and have examined the distribution of these proteins in hybrid myotubes in which one or only a few nuclei express the protein. Two segments of the glucocorticoid receptor contain nuclear localization signals (*6*). When fused to *Escherichia coli* β-galactosidase, one of these, NL1, targets the protein to the nucleus in the absence of glucocorticoid, whereas the other, NL2, targets the protein to the nucleus only in the presence of hormone (*6*). We have used these two fusion proteins to determine the distribution of both targeted and untargeted proteins produced from a single nucleus within the myotube.

The plasmid for each fusion protein was transfected into C2 myoblasts (*7*), along with ptk-neo, a plasmid conferring neomycin resistance (*8*). Clones of transfected cells were selected by resistance to the neomycin analog G418; those with the highest expression of each fusion protein were identified by immunofluorescence and were subcloned to form stable lines. The selected lines were designated C2-Zn (targeted to the nucleus in the absence of hormone) or C2-Zc (cytoplasmic in the absence of hormone).

When the protein was localized in myoblast cultures by immunofluorescence (Fig. 1, left), β-galactosidase was found mostly in the nuclei of C2-Zn myoblasts, but only in the cytoplasm of C2-Zc myoblasts. Addition of the glucocorticoid dexamethasone to cultures of C2-Zc myoblasts, however, resulted in the rapid transport of the fusion protein to the nucleus. No signal was detected when the untransfected myoblasts were stained. Although each cell in cultures of the C2-Zc and C2-Zn clones was clearly stained, there was some variability in the amount of fluorescence between cells within a clonal line.

After transfer to low-serum medium, both C2-Zn and C2-Zc cells fused to form multinucleated myotubes that were indistinguishable from those formed by untransfected cells. The distribution of fusion protein in the myotubes (Fig. 1, right) was similar to that seen with myoblasts. The protein was localized mostly in the nuclei of C2-Zn myotubes and in the cytoplasm of C2-Zc myotubes, but in the latter case was transported to the nuclei on the addition of dexamethasone to the culture medium.

Hybrid myotubes were formed between each of the transfected cell lines and the parental C2 myoblasts (*9*). To obtain hybrid myotubes with one or a few labeled nuclei, we used a 20:1 ratio of parental to transfected myoblasts. The cells were plated at high density to favor immediate fusion and to avoid the formation of small segregated clones of each cell line. To identify the source of the individual nuclei, we incubated the transfected cells in [^{3}H]thymidine before fusion. In control experiments, myotubes were formed with only the labeled cells (Fig. 2). Examination of 359 nuclei in three independent experiments showed that 99% of the nuclei identified with the dye Hoechst 33258 were radioactively labeled.

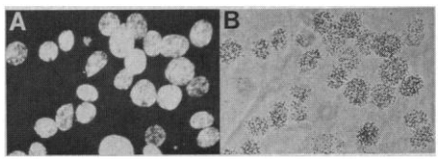

Fig. 2. Labeling of C2 nuclei with [^{3}H]thymidine. C2 myoblasts, grown with [^{3}H]thymidine (*9*), were trypsinized and plated on four-chamber slides at a density of 60,000 cells per square centimeter. After 3 days in fusion medium, they were fixed, stained with bis-benzimide (Hoechst 33258) (Sigma), and coated with emulsion (Ilford K5). After 2 weeks the slides were developed and mounted. (**A**) All nuclei are visible under ultraviolet fluorescence after staining with Hoechst 33258. (**B**) The same field under phase optics shows that essentially all nuclei can be identified by ^{3}H autoradiography.

Thus, nuclei from the transfected cells could be identified with certainty.

When hybrid myotubes formed by using radioactively labeled Zn myoblasts and unlabeled parental myoblasts were examined by autoradiography and immunofluorescence, it was apparent that the distribution of the fusion protein usually extended beyond the nucleus encoding it. Thus, in 17 of 20 cases examined, not only the nucleus labeled with [^{3}H]thymidine, but also nearby unlabeled nuclei were positive for β-galactosidase (Fig. 3, A to F). For each of these 20 ^{3}H-labeled nuclei, an average of 3.8 ± 2.2 nuclei (mean ± SD), including the source nucleus, were stained for β-galactosidase. In one instance, a cluster of ten nuclei surrounding a single radioactive nucleus were all positive for the fusion protein (Fig. 3D). The intensity of nuclear staining for β-galactosidase progressively decreased with distance from the nucleus encoding it, so that nuclei more than a few nuclear diameters away were unstained. The extent of staining, measured from the center of the source nucleus to the center of the furthest stained nucleus, averaged 34 ± 31 μm. Nuclei in the immediate vicinity were not always stained (Fig. 3E), and direct contact with the source nucleus was not required to observe spreading of the fusion protein (Fig. 3F). We conclude that a protein targeted to the nucleus is not confined to the source nucleus, but may spread to other nuclei in its immediate vicinity.

Hybrid myotubes with C2-Zc cells and the parental C2 cells behaved quite differently. In the absence of dexamethasone, faint staining was seen throughout the cytoplasm, indicating that the protein was not restricted in its diffusion along the length of the myotube. When hormone was applied 1 hour before fixation and staining, many β-galactosidase–positive nuclei were observed. In contrast to myotubes formed with C2-Zn

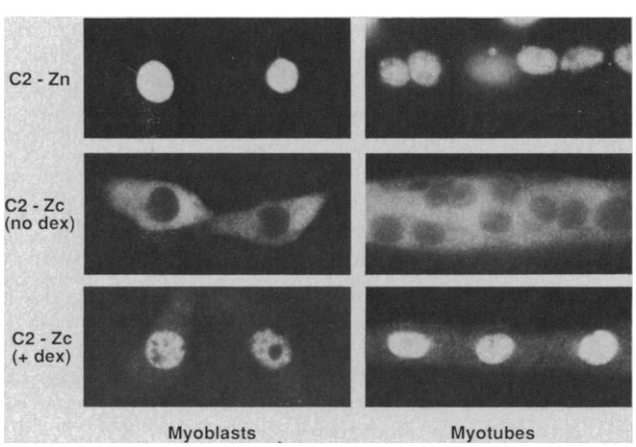

Fig. 1. Expression of fusion proteins in transfected C2 cells. Transfected C2 myoblasts (left) or myotubes (right), grown on glass cover slips, were fixed at room temperature with 2% paraformaldehyde for 20 min, permeabilized with 1% Triton X-100 in Dulbecco's phosphate-buffered saline (PBS), stained with a mouse monoclonal antibody to β-galactosidase (Promega), followed by a fluorescein isothiocyanate (FITC)–labeled goat antibody to mouse immunoglobulin G (IgG) (Cappel Labs) Cover slips were mounted in 90% glycerol containing paraphenylenediamine (*17*). Cells were observed with a Leitz Ortholux II fluorescence microscope equipped with a Vario-Orthomat camera system. Dexamethasone (dex) (10^{-6} *M* final concentration, obtained from a 10^{-4} *M* stock solution in ethanol) was added 1 hour before fixation as indicated.

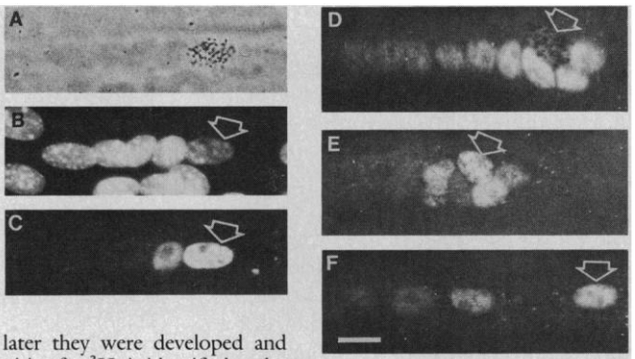

Fig. 3. Distribution of fusion protein in hybrid myotubes containing Zn nuclei. Hybrid myotubes (*9*) were formed between untransfected C2 myoblasts and ^{3}H-labeled Zn myoblasts, in a ratio of 20:1. After 3 days in fusion medium, cells were fixed, permeabilized, and stained for β-galactosidase. Nuclei were stained with Hoechst 33258 and the slides were processed for autoradiography. Two weeks later they were developed and mounted. (**A**) A Zn nucleus, positive for ^{3}H, is identified under phase optics. (**B**) The same field is observed under fluorescence optics for the Hoechst dye to reveal other nuclei in the same and in neighboring myotubes. (**C**) The same field is observed under fluorescence with the filter for FITC to reveal those nuclei that contain detectable levels of β-galactosidase. (**D** to **F**) Other examples of β-galactosidase staining in fields containing one ^{3}H-labeled nucleus. In each case, the same steps were followed as in (A) to (C) to identify the nucleus and ascertain that it was included in a mature myotube. The single ^{3}H-labeled nucleus is indicated by the arrowhead in (B) to (F). Bar, 20 μm.

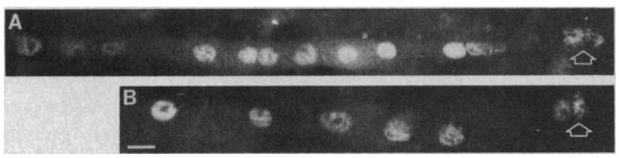

Fig. 4. Distribution of fusion protein in two hybrid myotubes (**A** and **B**) containing Zc nuclei. Experiments were performed as in Fig. 3, except that Zc myoblasts were used instead of Zn myoblasts. Dexamethasone (10^{-6}*M*) was added 1 hour before fixation of the cells. The arrowheads indicate the ^{3}H-labeled nucleus. Bar, 20 μm.

cells, positive nuclei could be easily detected at least 400 μm from the source nucleus (Fig. 4, A and B). In 17 myotubes, each containing one ^{3}H-labeled nucleus, we found an average of 10.3 ± 4.6 β-galactosidase–positive nuclei for each ^{3}H-labeled nucleus; the furthest extent of staining averaged 224 ± 97 μm. These numbers are probably underestimated, as quantitative estimates were limited by the difficulty of following single fibers for long distances.

The results obtained with hybrid myotubes containing one or a few C2-Zc nuclei are consistent with experiments showing that proteins are free to diffuse within the myotube cytoplasm (*10*). The Zc fusion protein has a molecular size of about 160 kD and probably forms a tetramer (*11*). Our results thus suggest that there are no barriers to the movement of even quite large proteins within the cytoplasm of the myotube.

In hybrid myotubes formed with C2-Zn myoblasts, the fusion protein was localized to the source nucleus and its neighbors. Thus factors produced from one nucleus can be taken up by others. Blau *et al.* (*5*) have postulated that such factors are responsible for the activation of muscle genes from human fibroblast nuclei in heterokaryons. Our experiments demonstrate directly that the products of one muscle nucleus within a myotube can interact with others.

In myotubes containing a single (or a few) Zn nuclei, the fusion protein was not found in nuclei throughout the fiber, but only in those in the immediate vicinity. In general, labeled nuclei were within 50 μm of the source nucleus; none were found more than 100 μm away. This observation leads to several conclusions. (i) The stained nuclei must have a relatively constant relation to one another within a time scale defined by the half-life of the fusion protein. Thus, if nuclear movement occurs in C2 myotubes, it must be slow or rare compared to the displacement of 15 μm per hour reported for chick myotubes (*12*). (ii) Neither mRNA nor polysomes migrate a long distance from their site of origin in myotubes. Although earlier experiments (*13*) have suggested that mRNA is not confined to the region around the nucleus of origin, our results indicate that mRNA does not range far. A similar inference can be drawn from experiments showing that AChR mRNA is found around subsynaptic nuclei (*4*). (iii) Binding of the fusion protein by nearby nuclei must be relatively efficient and must occur at a rapid rate compared to the time required for the protein to diffuse along the length of the fiber (*14*). (iv) In studies of muscle lineage, caution must be exercised in the use of reporter proteins directed to the nucleus (*15*), since proteins produced by one nucleus in a myotube may spread to its neighbors.

The major implication of our experiments, however, is the suggestion that single nuclei may have local domains of influence within a myotube. Previous experiments on chimeric mice in which electrophoretically distinct isoenzymes are produced from different nuclei demonstrated that products of each type of nucleus were uniformly distributed within the fibers (*13*), leading to the conclusion that nuclei do not have distinct domains within myotubes. If the nuclei were randomly distributed, however, these experiments would not have detected domains extending over several nuclear diameters. By examining specifically the products of single identified nuclei, we have been able to demonstrate that a protein targeted to the nucleus has only a limited range.

Note added in proof: After submission of this manuscript, a report appeared (*16*) that also suggests the existence of nuclear domains within myotubes.

REFERENCES AND NOTES

1. S. W. Kuffler, *J. Neurophysiol.* **6**, 99 (1943); H. C. Fertuck and M. M. Salpeter, *J. Cell Biol.* **69**, 144 (1976).
2. G. Salviati *et al.*, *Nature* **332**, 637 (1986).
3. J. P. Merlie and J. R. Sanes, *ibid.* **317**, 66 (1985).
4. B. Fontaine *et al.*, *EMBO J.* **7**, 603 (1988).
5. H. M. Blau, C.-P. Chiu, C. Webster, *Cell* **32**, 1171 (1983); H. M. Blau *et al.*, *Science* **230**, 758 (1985).
6. D. Picard and K. R. Yamamoto, *EMBO J.* **6**, 3333 (1987).
7. Cultures of C2 muscle cells [D. Yaffe and O. Saxel, *Nature* **270**, 725 (1977)] were maintained as follows. Growth medium was DME H-16 with glucose (1 g/liter), supplemented with 0.5% chick embryo extract (Gibco), 20% fetal bovine serum, 2 m*M* L-glutamine, 100 units of penicillin per milliliter, and 100 μg of streptomycin per milliliter. Fusion medium was DME H-16 supplemented with 5% horse serum and 2 m*M* L-glutamine. The cells were incubated at 37°C in 8% CO_2. When fusion was apparent, the medium was supplemented with 10^{-5}*M* cytosine β-arabinoside (Ara C) for 24 hours to kill unfused myoblasts.
8. The plasmids encoding the fusion proteins are described in (*6*). They encode fusion proteins with amino acids 497 to 524 (NL1) and 407 to 794 (NL2), respectively, of the rat glucocorticoid receptor fused to *E. coli* β-galactosidase. The vector also contains an SV40 enhancer and the human α1-globin promoter (*6*). Transfections were performed with the calcium phosphate method [F. L. Graham and A. J. van der Eb, *Virology* **52**, 456 (1973); M. Wigler, A. Pellicer, S. Silverstein, R. Axel, *Cell* **14**, 725 (1978)]. Twenty-four hours after C2 myoblasts were plated at 2×10^5 to 5×10^5 cells per 10-cm dish, the medium was replaced by 1.5 ml of a calcium phosphate precipitate containing 20 μg of Zc or Zn plasmid and 2 μg of ptk-neo. After 30 min at room temperature, with occasional swirling, growth medium was added and the cells returned to the incubator. Five to 12 hours later, the medium was replaced by 5 ml of 15% glycerol in serum-free growth medium. After 1 min, the cells were rinsed with Dulbecco's PBS and returned to growth medium. Twenty-four hours later, the cells were split into several dishes at 8×10^4 cells per 10-cm dish and incubated in growth medium supplemented with 500 μg (active) of G418 (Gibco) per milliliter. After 10 to 12 days, G418-resistant colonies were isolated by trypsinization within small glass cloning rings.
9. The parental C2 cells and C2-Z cells (C2-Zc or C2-Zn) were plated on two separate 10-cm dishes at 7.5×10^4 cells per dish. On the following day, fresh growth medium was added and the C2-Z cells were supplemented with [^{3}H]thymidine (ICN Radiochemicals; 6.7 Ci/mmol) at a final concentration of 0.05 μCi/ml. Higher concentrations of [^{3}H]thymidine were toxic [U. K. Ehmann *et al.*, *Nature* **258**,

633 (1975); M. Hirayama, P. A. Eccleston, D. H. Silberberg, *Dev. Biol.* **104**, 413 (1984)]. Forty-eight hours later, the cells were trypsinized and plated together at a density of 60,000 cells per square centimeter on the wells of duplicate Lab-Tek four-well slides (Nunc Labs) that had been treated with a 1:5 dilution of Vitrogen (Collagen). Twenty-four hours later, fusion medium was added. On the next day, fusion medium supplemented with Ara C was added, and 1 to 2 days later the cells were processed for immunocytochemistry.

10. J. R. Blinks, R. Rudel, S. R. Taylor, *J. Physiol. (London)* **277**, 291 (1977).
11. Enzymatically active β-galactosidase can be detected in both Zc and Zn cells by using X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) as substrate. Because *E. coli* β-galactosidase must be at least tetrameric to be active [I. Zabin and A. N. Fowler, in *The Operon*, J. H. Miller and W. S. Reznikoff, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1980), pp. 89–121], Zc and Zn fusion proteins probably assemble as tetramers.
12. L. L. Englander and L. L. Rubin, *J. Cell Biol.* **104**, 87 (1987).
13. B. Mintz and W. W. Baker, *Proc. Natl. Acad. Sci. U.S.A.* **58**, 592 (1967); P. M. Frair, P. M. Strasberg, K. B. Freeman, A. C. Peterson, *Biochem. Genet.* **17**, 693 (1979); P. M. Frair and A. C. Peterson, *Exp. Cell Res.* **145**, 167 (1983).
14. If one assumes a diffusion constant (20°C, H_2O) of about 10^{-7} cm^2 s^{-1} for a globular protein of 500,000 kD [C. R. Cantor and P. R. Schimmel, *Biophysical Chemistry, Part II* (Freeman, San Francisco, 1980), p. 584], one can calculate by Einstein's equation, $x^2 = 2\,Dt$ (where x is the distance, D is the diffusion constant, and t is time), that the β-galactosidase fusion protein Zn would diffuse 50 μm in about 2 min. If the effective diffusion constant in cytoplasm is reduced ten-fold (*10*), the corresponding time would be increased to 20 min.
15. C. Bonnerot *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6795 (1987).
16. G. K. Pavlath, K. Rich, S. G. Webster, H. M. Blau, *Nature* **337**, 570 (1989).
17. J. L. Platt and A. F. Michael, *J. Histochem. Cytochem.* **31**, 840 (1983).
18. We thank D. Picard and K. Yamamoto for the plasmids encoding the fusion proteins and for advice and encouragement; N. Fregien for the plasmid ptk-neo; H. Gordon for his help in devising the [^{3}H]thymidine labeling procedure; H. Blau for helpful early discussions; and the members of the laboratory and other colleagues for constructive comments on the manuscript. Supported by grants from NIH (grant NS 20107) and the Muscular Dystrophy Foundation.

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Hidden Thermodynamics of Mutant Proteins: A Molecular Dynamics Analysis

JIALI GAO, KRZYSZTOF KUCZERA, BRUCE TIDOR, MARTIN KARPLUS

A molecular dynamics simulation method is used to determine the contributions of individual amino acid residues and solvent molecules to free energy changes in proteins. Its application to the hemoglobin interface mutant Asp G1(99)β→Ala shows that some of the contributions to the difference in the free energy of cooperativity are as large as 60 kilocalories (kcal) per mole. Since the overall free energy change is only −5.5 kcal/mole (versus the experimental value of −3.4 kcal/mole), essential elements of the thermodynamics are hidden in the measured results. By exposing the individual contributions, the free energy simulation provides new insights into the origin of thermodynamic changes in mutant proteins and demonstrates the role of effects beyond those usually considered in structural analyses.

CHANGES IN PROTEIN STABILITY and function resulting from mutations can be determined experimentally. The alterations in free energy are often interpreted in terms of the structures of the wild type and mutant protein (*1, 2*). However, the connection between structure and measured thermodynamic quantities is not simple; for example, relatively small measured free energy changes can be made up of large contributions from a number of protein-protein and protein-solvent interactions that almost cancel. Moreover, the consequences of the removal of repulsive interactions, not usually considered in structural studies, can be as important as the loss of attractive terms (such as hydrogen bonding). We demonstrate how molecular dynamics (MD) free energy simulations can be used to estimate the individual thermodynamic contributions that result from a mutation. The change in the free energy of cooperativity of a mutant of hemoglobin [Asp G1(99)β→Ala] is decomposed into contributions from the solvent, intersubunit interactions, and intrasubunit alterations. The qualitative insights obtained from this decomposition increase our understanding of protein mutants since the effects described for hemoglobin are expected to play a general role.

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The free energy difference ΔG between two states A and B can be calculated by Monte Carlo and MD techniques (*3–7*). If A corresponds to the wild-type protein and B to the mutant, a simulation (which can be envisioned as "computer alchemy" since one amino acid is transformed into another) can be used to determine their free energy difference. The simulation is based on thermodynamic integration (*8*) with the equation

$$\Delta G = G_B - G_A = \int_0^1 \langle \Delta V \rangle_\lambda d\lambda \simeq \sum_i \langle \Delta V \rangle_{\lambda_i} \Delta\lambda \quad (1)$$

where $\Delta V = V_B - V_A$ and λ is a parameter, such that $V_\lambda = (1 - \lambda)V_A + \lambda V_B$ (*9*); V_A and V_B are empirical energy functions describing the solvated normal and mutant hemoglobin molecules, respectively. The calculation uses MD for evaluation of the thermodynamic average, $\langle \Delta V \rangle_\lambda$, where the subscript λ indicates that the average is over the hybrid system described by V_λ. The linear form of Eq. 1 allows the free energy to be decomposed as

$$\Delta G = \Delta G_0 + \sum_{r>0} \Delta G_r \quad (2)$$

where ΔG_0 is the contribution from the free energy change of the mutated residue and ΔG_r ($r > 0$) are the contributions from interactions between the mutated residue and any other residue or water molecule.

The free energy simulation and decomposition method is illustrated for hemoglobin, the classic example of cooperativity (*10–12*). We consider the $\alpha_1\beta_2$ interface residue Asp G1(99)β for which a series of naturally occurring mutants have been shown to have significantly reduced cooperativity and increased oxygen affinity relative to normal hemoglobin (*10, 11, 13*). Based on x-ray structures of deoxy and oxy hemoglobin A, Perutz *et al.* (*14–16*) suggested that the essential role of Asp G1(99)β is to stabilize the deoxy tetramer by making hydrogen bonds to Tyr C7(42)α and to Asn G4(97)α; these hydrogen bonds are absent in the oxy tetramer. We supplement this observational conclusion by a theoretical analysis of the free energy changes induced by the mutation Asp G1(99)β→Ala [Hb Radcliffe (*17*)], which is of the "deletion" type (*1*), and is therefore expected to lead to relatively localized structural changes (*18*) that are simplest to interpret (*19*). The overall change in the free energy of cooperativity (*13*) is shown to result from a set of large but nearly compensating interactions of Asp G1(99)β with several amino acids and with the solvent. Although both Tyr C7(42)α and Asn G4(97)α play a significant role, the calculations indicate that the solvent and other interchain and intrachain interactions are also important.

The essential free energy simulation results are shown in Table 1 (*9, 21, 22*). Energy-minimized unliganded (*23*) and liganded (*24*) x-ray structures for the normal hemoglobin tetramers were used to generate the starting configurations (Fig. 1), and the stochastic boundary molecular dynamics (SBMD) method (*25*) was used for the $\langle \Delta V \rangle_\lambda$ simulations (*26–28*). Both the deoxy and oxy tetramers are destabilized by the mutation (by 66 and 60.5 kcal/mole, respectively, per $\alpha_1\beta_2$ interface) (*29*), but the deoxy tetramer is more destabilized, leading to the observed reduction in cooperativity