Mkn 348, but its location at the end of the outermost, bright optical arm fits the configuration predicted by the computer model of Appleton *et al.* Finally, the continuous H I arms displayed in Fig. 1B and the observed reversals in the H I velocities are also consistent with this computer model. In combination, these observations provide strong evidence that Mkn 348 has been subjected to tidal perturbations and should therefore be a good candidate for further study of how such perturbations influence its active galactic nucleus. Additional H I measurements are needed to establish the link between NGC 266 and NGC 262, if any. Because Mkn 348 presents a unique opportunity to study the mass in the far outer regions of a galaxy by means of assumptions very different from those involved in the statistical studies of galaxy groups and pairs, it is also essential that these observations be followed by more detailed computer models of this system.

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Mutants of Bovine Pancreatic Trypsin Inhibitor Lacking Cysteines 14 and 38 Can Fold Properly

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It is a generally accepted principle of biology that a protein's primary sequence is the main determinant of its tertiary structure. However, the mechanism by which a protein proceeds from an unfolded, disordered state to a folded, relatively well-ordered, native conformation is obscure. Studies have been initiated to examine the "genetics" of protein folding, with mutants of bovine pancreatic trypsin inhibitor (BPTI) being used to explore the nature of the specific intramolecular interactions that direct this process. Previous work with BPTI chemically modified at cysteines 14 and 38 indicated that transient disulfide bond formation by these residues contributed to efficient folding at 25°C. In the present work, mutants of BPTI in which these cysteines were replaced by alanines or threonines were made and the mutant proteins were produced by a heterologous *Escherichia coli* expression system. At 25°C in vitro, the refolding behavior of these mutants was characterized by a pronounced lag. However, when expressed at 37°C in *E. coli*, or when refolded at 37° or 52°C in vitro, the mutant proteins folded readily into the native conformation, albeit at a rate somewhat slower than that exhibited by wild-type BPTI. These results indicate that, at physiological temperatures, BPTI lacking cysteines 14 and 38 can refold quantitatively.

BOVINE PANCREATIC TRYPSIN INHIBitor (BPTI) is a 58–amino acid basic polypeptide that has three disulfide bonds: Cys14/Cys38, Cys30/Cys51, and Cys5/Cys55. When it is refolded from the fully reduced form in vitro, it proceeds to its native form via a series of preferred one- and two-disulfide intermediates (*1, 2*). Cysteines 14 and 38, which form a disulfide bond in the native molecule, participate in disulfide bonding in the most abundant two-disulfide intermediates (*1, 3*). Two of these two-disulfide bond intermediates contain non-native disulfide bonds, Cys14/Cys5 or Cys38/Cys5, in addition to the disulfide Cys30/Cys51. Creighton (*1*) showed that when cysteines 14 and 38 were blocked by reduction and alkylation, the modified BPTI could not readily regain its native conformation when refolded in vitro at 25°C. These results suggested that non-native disulfide bonds involving cysteines 14 and 38 were components of a highly preferred pathway for BPTI refolding (*1, 3*).

The above studies were open to the objection that the bulky alkylating agents used to block cysteines 14 and 38 sterically interfered with refolding. We therefore decided to repeat this experiment with genetically modified BPTIs in which cysteines 14 and 38 were replaced by alanines or threonines. The mutant BPTI proteins were synthesized by means of a heterologous expression and secretion system in *Escherichia coli* that produces native, correctly folded BPTI (*4*).

The [Ala14,Ala38]BPTI and [Thr14, Thr38]BPTI mutants were expressed by *E.*

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coli at levels comparable to the level of wild-type BPTI expression. Amino acid analyses of the purified mutant proteins yielded compositions that were consistent with alanine or threonine substitutions. The number of cysteines per polypeptide chain was also measured directly. The proteins were fully reduced with dithiothreitol in the presence of urea, alkylated with a mixture of iodoacetate and iodoacetamide, and analyzed by gel electrophoresis (*5*). Four cysteines were found in each mutant protein (Fig. 1). The gel results and the amino acid compositions also indicated that cleavage of the signal sequence from the mutant preproteins had occurred, similar to the processing observed for wild-type BPTI secreted from *E. coli* (*4*).

To assess whether intact native disulfides were present, we treated mutant and wild-type BPTIs with iodoacetate, dithiothreitol, or urea (Table 1) (*4*). These treatments have been used previously to detect BPTI refolding intermediates in which particular disulfide bonds were missing (*3, 4*). The mutant proteins were resistant to alkylation by iodoacetate in the presence of 8*M* urea (Fig. 2), indicating that no free thiols were present (*3*). The mutant proteins were also resistant to reduction by dithiothreitol (Fig. 2), indicating that although the Cys14/Cys38 disulfide was missing, the other two native disulfides were intact (*1, 3*). The presence of the Cys30/Cys51 and Cys5/Cys55 disulfide bonds was confirmed by two-dimensional thin-layer chromatography (*6*).

By several other criteria the mutant proteins appeared to have a native-like structure similar to BPTI that had been selectively reduced at the Cys14/Cys38 disulfide and alkylated with iodoacetamide ([14-cam,38-cam]BPTI). The mutants migrated with the same mobility on polyacrylamide-urea gels (*7*) as [14-cam,38-cam]BPTI, and they bound trypsin stoichiometrically (*8, 9*). The mutant BPTIs exhibited thermal denaturation transitions in the same temperature range (60° to 62°C at *p*H 2.2) as that of [14-cam,38-cam]BPTI (*10, 11*). Two-dimensional nuclear magnetic resonance (NMR) spectra of the mutant proteins also indicated that, like [14-cam,38-cam]BPTI, they had conformations similar to native BPTI, with chemical shift differences localized in the regions near residues 14 and 38 (*10, 12*). In summary, these data showed that the [Ala14,Ala38]BPTI and [Thr14,Thr38] BPTI mutants synthesized by *E. coli* were closely related to native BPTI in conformation, indicating that at least under some conditions in vivo BPTI does not need cysteines 14 and 38 in order to fold properly.

To explore their properties further, we studied the refolding of the mutant proteins in vitro. The proteins were fully reduced, then allowed to refold in the presence of oxidized dithiothreitol or reduced and oxidized glutathione. At various times the folding reactions were quenched with an excess of iodoacetamide, and trypsin inhibition assays were performed to measure regain of the native conformation. Misfolded BPTI molecules with incorrect disulfide bonds, as well as molecules with reduced Cys5/Cys55 or Cys30/Cys51 disulfide bonds, are inactive against trypsin when modified by alkylation (*1, 3, 13*). Therefore, the iodoacetamide quenching procedure followed by the activity assay effectively discriminated between native-like molecules, in which the Cys5/Cys55 and Cys30/Cys51 disulfide bonds were present, and those with a non-native disulfide bond configuration (*14*). Comparable results were obtained when the polyacrylamide gel method of Creighton and Goldenberg (*3*) was used to monitor refolding (*8*).

The [Ala14,Ala38]BPTI did not refold at 25°C in 25 minutes in the presence of oxidized dithiothreitol, in agreement with the previous results of Creighton for [14-cam,38-cam]BPTI (*1*). However, [Ala14, Ala38]BPTI did refold at 37°C in the presence of oxidized dithiothreitol (*6*). In the presence of the more potent oxidant, glutathione, the [Ala14,Ala38]BPTI and [Thr14,Thr38]BPTI mutants refolded approximately three times more slowly than wild-type BPTI at 37°C (Fig. 3); [14-cam,38-cam]BPTI behaved similarly (*15*). Despite the decrease in the rate of refolding, all three of the modified BPTIs could nevertheless readily regain the native conformation. Alanine has a small nonpolar side chain, carboxyamidomethylcysteine a bulky linear side chain, and threonine a branched polar side chain, yet within the limits of experimental error all three of the substitutions yielded molecules with identical folding kinetics. These results strongly suggest that the observed retardation of folding is due primarily to the absence of cysteines 14

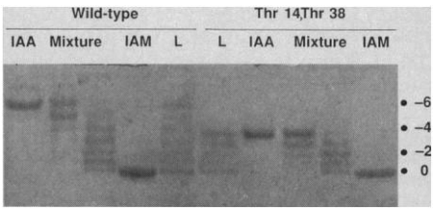

Fig. 1. Quantitation of cysteines in wild-type BPTI and [Thr14,Thr38]BPTI. The number of cysteines in wild-type BPTI and the Thr14,Thr38 mutant were compared by the method of Creighton (*5*). Wild-type (Trasylol, Bayer AG) and mutant BPTIs were treated, at a concentration of 250 μg/ml, with 10m*M* tris-HCl (*p*H 8.0), 1 m*M* EDTA, 10 m*M* dithiothreitol, and 8*M* urea for 30 minutes at 37°C to fully reduce and denature the molecules. The free thiols were then blocked by adjusting the samples to a final concentration of 50 m*M* iodoacetate, 50 m*M* iodoacetamide, or 50 m*M* mixtures of these two alkylating agents in various proportions. Alkylations proceeded at 25°C for 60 minutes in the dark. Alkylated BPTI molecules containing different charges were then resolved by electrophoresis on a 15% acrylamide–8*M* urea gel (*5, 6*). The heterogeneity engendered in the population by the mixed alkylation resulted in a "ladder" of the variously charged species on the gel, enabling the number of cysteines per polypeptide to be determined by inspection. Gels were stained in 10% trichloroacetic acid, 10% sulfosalicylic acid, 0.1% Coomassie blue (*5*). Lanes: IAA, iodoacetate; IAM, iodoacetamide; mixture, two samples from alkylation reactions containing various proportions of iodoacetate and iodoacetamide (42 m*M* IAA + 8 m*M* IAM and 25 m*M* IAA + 25 m*M* IAM); L, ladder produced by pooling samples from each reaction. Each lane represents approximately 2 μg of total protein. Numbers in the margin represent the relative charge differences (negative electrode at the bottom of the gel). Similar results were found for the Ala14,Ala38 mutant (*21*).

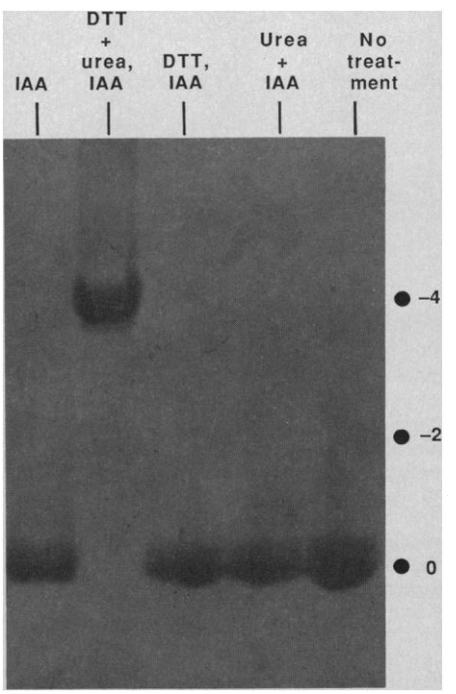

Fig. 2. Chemical probing of the disulfide status in the [Thr14,Thr38]BPTI mutant. The treatments described in Table 1 were carried out on the Thr14,Thr38 mutant; the results of such treatments on wild-type BPTI (summarized in Table 1) were described previously (*4*). A solution (250 μg/ml) of the mutant protein in 10 m*M* tris-HCl (*p*H 8.0) containing 1 m*M* EDTA was incubated in the presence of 8*M* urea, 10 m*M* dithiothreitol (DTT), or both, for 30 minutes at 37°C. Alkylations were performed at 25°C for 60 minutes in the presence of 50 m*M* iodoacetate (IAA). Samples from each reaction containing 5 μg of protein were analyzed by gel electrophoresis as described in Fig. 1. The charge difference corresponding to the observed mobility shift is shown in the margin.

Table 1. Susceptibility of correctly folded [Thr14,Thr38]BPTI to urea and dithiothreitol (DTT). Shown are the expected results for alkylations performed as described by Marks *et al.* (*4*). Actual results for wild-type and [Thr14, Thr38]BPTI are given in (*4*) and in Fig. 2, respectively. The conditions used were similar to those described by Creighton (*1*) and Creighton and Goldenberg (*3*). IAA, iodoacetate.

Treatment	Number of alkylated cysteines	
	Wild type	Thr14, Thr38
IAA	0	0
Urea + IAA	0	0
DTT, then IAA	2	0
DTT + urea, then IAA	6	4

and 38 rather than the exact chemical nature of the substitution.

To investigate the temperature dependence of the refolding rates, we also conducted refolding reactions at 52° and 25°C. At 52°C all the forms of BPTI refolded faster than at 37°C, but the rates of mutant refolding relative to wild-type BPTI were similar to the results observed at 37°C (Fig. 3). At 25°C, however, the mutant BPTIs showed a pronounced lag in their refolding behavior (Fig. 3), similar to that reported for BPTI alkylated at cysteines 14 and 38 (*1*).

Previous refolding studies on BPTI in vitro revealed two-disulfide intermediates having non-native disulfide bonds between cysteine 5 and cysteines 14 or 38. Furthermore, two other predominant two-disulfide intermediates ([Cys14/Cys38, Cys5/Cys55] and [Cys14/Cys38, Cys30/Cys51]) contain the native 14-38 pairing (*1, 3*). Our results with genetically and chemically altered BPTIs, in which cysteines 14 and 38 have been replaced or modified, extend and clarify these earlier observations. First, our results show that when cysteines 14 and 38 are not present, BPTI readily folds into its active native conformation at 37°C, both in vitro and when expressed in a heterologous system in vivo. Second, our results show that the refolding of BPTI molecules that are unable to form the Cys14/Cys38 disulfide bond is strongly temperature dependent. During refolding of the mutant and modified BPTIs at a low temperature, molecules with native disulfide bonds and an active conformation form only after an initial lag phase, whereas at physiological temperatures or above the mutants refold without a significant lag. Finally, there are no discernible differences in the refolding behavior of BPTI alkylated at cysteines 14 and 38 and BPTI in which alanine or threonine have been substituted for these residues.

Our results with BPTI at 37°C are similar to the refolding data for the homologous snake proteins, mamba inhibitors I and K, at 25°C (*16*). Inhibitors I and K with cysteines 14 and 38 blocked by alkylation readily refold at 25°C, albeit at a rate severalfold slower than the rates for unmodified inhibitors. The refolding of the alkylated proteins apparently occurs via the direct formation of the Cys30/Cys51 and Cys5/Cys55 disulfides without the participation of non-native disulfide bonds (*16*). Temperatures around 25°C are physiological for reptiles but not for mammals. Therefore, homologous proteins such as BPTI and inhibitors I and K may have evolved to have similar, and presumably optimal, folding properties at or near their respective physiological temperatures. One of these properties may be an allowance for a multiplicity of folding paths (*17, 18*).

Among the many types of interatomic interactions that stabilize protein tertiary structures, the only covalent bonds that are commonly found are disulfides. A necessary and sufficient condition for the formation of a protein disulfide is that, in the presence of an appropriate oxidant, two cysteines are brought together with the correct relative geometry and spacing by the folding of the polypeptide chain. It is meaningful to ask, therefore, whether disulfide bonds merely serve to mark chain interactions that are driven by other forces, or whether the formation of disulfides can actually redirect the folding process. The roughly threefold decrease in the rate of refolding exhibited by the [Ala14,Ala38]BPTI and [Thr14, Thr38]BPTI mutants indicates that the overall activation energy barrier for the refolding reaction has been raised slightly (*15*). Similar decreases in the rate of refolding have been observed for bovine trypsinogen when disulfide bridges were removed by chemical modification (*19, 20*). These results, together with the data for reduced and alkylated mamba inhibitors (*16*), imply that the formation of at least some disulfide bonds can provide a modest driving force for protein folding. There is no reason to suppose, however, that all protein disulfides will have this property.

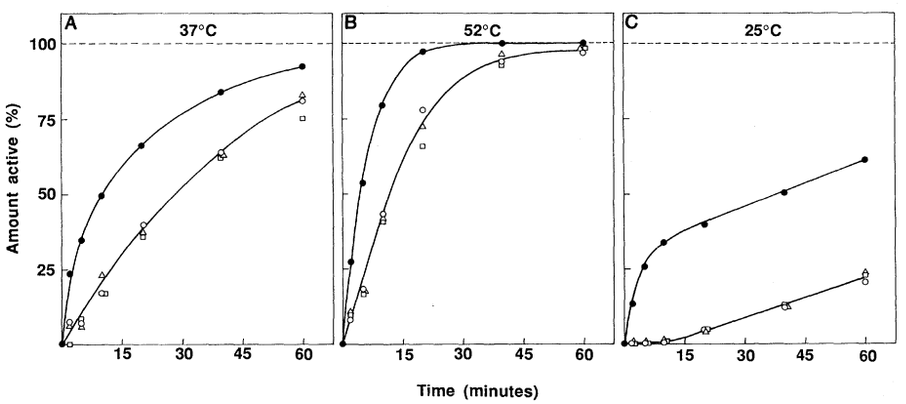

Fig. 3. Refolding in vitro of [Ala14,Ala38]BPTI, [Thr14,Thr38]BPTI, and [14-cam,38-cam]BPTI. Wild-type BPTI, the two mutants, and [14-cam,38-cam]BPTI [prepared as described by Creighton (*1*)] were refolded in vitro by a modification of the method of Creighton and Goldenberg (*3, 22, 23*). Trypsin inhibition assays were performed on the iodoacetamide-quenched samples to determine the amount of active, refolded BPTI that was present (*14*). Each sample was diluted 1:100 into a solution of 0.2*M* triethanolamine, 10 m*M* $CaCl_2$, 10 μg/ml bovine trypsin and incubated for 15 minutes at 25°C to allow trypsin:BPTI complex formation. *N*-α-benzoyl-DL-arginine-*p*-nitroanilide was then added to a final concentration of 0.4 mg/ml and the activity due to uncomplexed trypsin was measured by monitoring the change in absorbance at 405 nm as a function of time. Control experiments showed that the small amount of residual iodoacetamide present (<1 m*M*) did not inhibit the trypsin. (**A**) The percentage of the total BPTI present that was active against trypsin is plotted as a function of time of refolding at 37°C. Symbols: ●, wild type; ○, [Thr14,Thr38]BPTI; □, [Ala14,Ala38]BPTI; △, BPTI with cysteines 14 and 38 blocked by iodoacetamide alkylation. (**B** and **C**) Results of similar experiments performed at 52° and 25°C, respectively. For the wild-type refolding experiments, similar results were obtained with both natural BPTI (Trasylol, Bayer AG) and *E. coli*–derived material (*4*).

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22. The proteins were fully reduced by incubating them at a concentration of 600 μg/ml in 100 m*M* tris-HCl (*p*H 8.7), 1 m*M* EDTA, 8*M* urea, 10 m*M* dithiothreitol for 60 minutes at 37°C. The reduced proteins were then isolated by gel filtration on Sephadex G-25 in 10 m*M* HCl and used immediately. Refolding reactions were carried out in 100 m*M* tris-HCl (*p*H 8.7), 200 m*M* KCl, 1 m*M* EDTA, 10 m*M* reduced glutathione, 1 m*M* oxidized glutathione, and 30 μ*M* BPTI (*23*). Reactions were started by mixing together warmed solutions of reduced BPTI (in 10 m*M* HCl) and concentrated refolding buffer. Temperatures were maintained in heating blocks in the ranges 24° to 25°C, 37° to 38°C, and 51° to 53°C. At various times 40-μl aliquots were withdrawn, mixed with 10 μl of 0.5*M* iodoacetamide, and incubated at 25°C for 60 minutes in the dark to alkylate protein thiols. Iodoacetamide incubations in the presence of 8*M* urea, which should expose buried thiols (*3*), did not result in further modification of the protein (*8*), indicating that alkylation of all thiols was essentially complete under our conditions.
23. The *p*H of the refolding buffer was adjusted to 8.7 at room temperature before BPTI addition; the actual *p*H values of the refolding reactions were 8.3, 8.0, and 7.6 at 25°C, 37°C, and 52°C, respectively. Essentially the same results were obtained when the *p*H was maintained at 8.7 in all of the reactions (*8*), but the refolding rates were correspondingly faster at the higher *p*H [T. E. Creighton, *J. Mol. Biol.* **144**, 521 (1980)], and the lag time observed for the mutants at 25°C was reduced.
24. We thank B. Bochner and M. Brochier for help in producing the BPTI mutants, A. Kossiakoff for support and encouragement, and R. Drewes for advice on reptile body temperatures. This work was supported by Genentech, Inc. and grants from the NIH Institute of General Medical Sciences and Division of Research Resources.

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Superconductivity at 40 K in the Oxygen-Defect Perovskites $La_{2-x}Sr_xCuO_{4-y}$

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Structural, magnetic, and electronic properties of compounds in the series $La_{2-x}Sr_xCuO_{4-y}$ for $0.05 \leq x \leq 1.1$, with $\Delta x = 0.025$, were studied. Resistance, susceptibility, Meissner, and shielding measurements have revealed superconductivity among several members of the series. For $x = 0.15$, the transition temperature T_c, measured at the midpoint of the resistive transition, is a maximum at 39.3 K with a width of 2 K. At other compositions the transition is broader and occurs at a lower temperature. Annealing the $x = 0.15$ sample in oxygen at 500°C increases T_c to 40.3 K, while annealing at the same temperature under vacuum suppresses the superconductivity. These changes in oxygen content and T_c are reversible.

OXYGEN-DEFECT PEROVSKITES HAVE been studied for possible applications in electrocatalysis or oxygen sensing because they absorb oxygen. Some are also superconductors: the hexagonal alkali tungsten bronzes, with a critical temperature $T_c = 6$ K (*1*); $SrTiO_3$ doped with niobium, for which $T_c = 1$ K (*2*); and $BaPb_{1-x}Bi_xO_3$, for which $T_c = 13$ K (*3*). In all these metallic perovskites the conduction bands are made up largely of oxygen *p*-orbitals hybridized with metal *s*, *p*, or *d* orbitals (*4*). The nonintegral valence of the metal in these compounds is sometimes called mixed valence, a description that becomes more meaningful when the bands become narrow with respect to correlation or exchange energy.

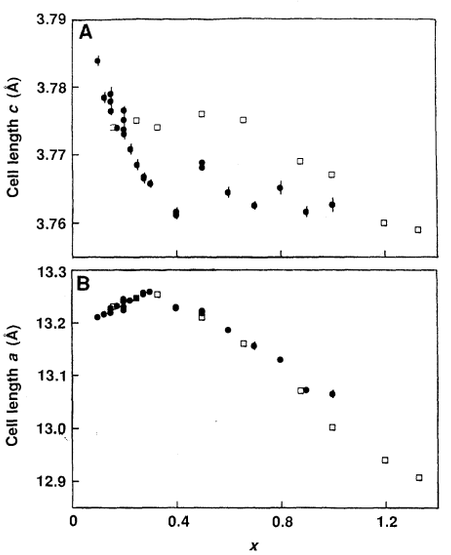

Fig. 1. Lengths *a* and *c* of the tetragonal unit cell as a function of strontium composition, *x*, in $La_{2-x}Sr_xCuO_{4-y}$; circles are our work and squares are from (*16*). Our error bars are twice the standard deviation from a least-squares refinement. For some *x*, more than one diffraction pattern was refined. For both $x = 0.15$ and $x = 0.200$ the highest value of *a* and the lowest value of *c* shown are for samples given different treatments than the rest. For $x = 0.15$ that sample was annealed under vacuum for 24 hours; for $x = 0.2$ that sample was annealed under oxygen and cooled in 10 hours. These different treatments did not change the lattice parameters by more than the scatter between the other points at those compositions.

Recently Bednorz and Müller (*5*) discovered evidence for much higher T_c (33 K) in the barium-lanthanum-copper (Ba-La-Cu) system. Shortly thereafter Bednorz *et al.* (*6*) and Ushida *et al.* (*7, 8*) independently confirmed superconductivity in the oxide $La_{2-x}Ba_xCuO_{4-y}$ with the K_2NiF_4 structure. Chu *et al.* (*9*) later reported an onset temperature for superconductivity of 52.5 K for this material under pressure.

This oxide is one of a large family of copper-oxide perovskites designated La-M-Cu-O, with M = Ba, Sr, or Ca, characterized by the presence of Cu^{+2} and Cu^{+3}. They were first synthesized by Longo and Raccah (*10*), and their structure and transport properties to 77 K were extensively studied by Nguyen *et al.* (*11, 12*). The superconductivity in the barium system prompted us to study the strontium analog $La_{2-x}Sr_xCuO_{4-y}$, which has been reported to be a solid solution for $0 < x < 1.1$ (*11*). [During the course of our work, other groups also observed bulk superconductivity in this system (*13*).] We show that the compound with $x = 0.15$ has $T_c = 39.3$ K and a transition width of 2 K, and we present evidence of sample inhomogeneity or phase segregation that broadens and lowers T_c at other compositions. We also show that T_c can be increased by annealing the compounds under oxygen, suppressed by annealing under vacuum, and that these changes in T_c with oxygen content are reversible. The highest T_c measured to date is on the $x = 0.15$ annealed sample, with a resistive midpoint of 40.3 K.

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