## Bovine Serum Albumin as a Catalyst. 5. A Sensitive Assay of the Correct Refolding of Individual Fragments of the Protein

*Sir:*

The results of both sequence analyses[1,2] and x-ray crystallography[3] indicate that a number of proteins are composed of distinct folded structural regions, or domains. Based on these observations and kinetic arguments Wetlaufer[3] has suggested that it is likely that in the early stages of the acquisition of three-dimensional structure, correct folding occurs independently within different domains of such proteins. He went on to suggest that a stringent test for independently folded domains would involve demonstrating that isolated fragments from proteins containing such domains can in fact fold properly.

The elegant sequence work of Brown,[1] coupled with Foster's[4] earlier studies indicates that bovine serum albumin (BSA) is a protein which is composed of at least three independently folded domains.[5] King[6] and later Feldhoff and Peters[7] have shown that it is possible to use pepsin digestion to prepare two high molecular weight fragments of BSA in very high purity. Fragment B, the N-terminal half of the molecule, contains residues 1–306. Fragment A, the C-terminal half, contains residues 307–581. Together these complementary fragments comprise the entire molecule, and each contains one full domain, plus half of a second domain, according to Brown's[1] sequence work. These fragments therefore provide an excellent model system for testing Wetlaufer's[3] prediction. In this communication we report the successful independent refolding and reoxidation of these fragments starting with the totally unfolded and reduced species.

We have used the unusual catalytic activity we previously reported for BSA[8] to monitor the refolding process. In the pH range of ca. 7–10 BSA accelerates by a factor of ca. $10^4$ the rate of decomposition of the Meisenheimer complex, 1,1-dihydro-2,4,6-trinitrocyclohexadienate. Our previous work indicates this activity is exquisitely sensitive to the conformational integrity of the protein.[8c] We have already shown that ca. 70% of the catalytic activity of the BSA monomer is regained if the *entire* molecule is unfolded, reduced, and then allowed to slowly air reoxidize and refold.[8c]

Catalytic assays on BSA and its fragments were performed using our published protocols.[8b] All assays were done in 0.1 M Tris (Tris(hydroxymethyl)aminomethane) HCl, pH 8.0, at 24 ± 1 °C. Fragments A and B were prepared according to the procedures of Feldhoff and Peters.[7] The fragments were found to be free of contamination when examined via polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol. In addition, our catalytic assay served as a secondary criterion of purity. This assay is valid because while neither fragment A nor fragment B has any detectable catalytic activity, when they are combined in solution at final concentrations of ca. 10 μM, ca. 35% of the full activity is recovered.[8a]

Unfolding of fragments A and B and reduction of their disulfide bonds was accomplished by preparing separate solutions of each of these fragments at ca. 10 mg/ml, in 0.1 M Tris HCl, pH 8.0, containing $10^{-3}$ M EDTA and 8 M urea. Mercaptoethanol was added to a final concentration of ca. 0.3 M, the pH was adjusted to 9.0, and the solutions were allowed to stand for 3 h. Air reoxidation and refolding of each fragment was accomplished by diluting the above solutions ca. 150-fold into 0.1 M Tris HCl, pH 8, and then allowing them to stand for 5 days with free access to air. In some cases the refolding milieu also contained the *"native"* [9] *complementary* fragment at ca. an equimolar final concentration. Subsequently the solutions were concentrated ca. 20-fold in an Amicon concentrator (PM-10 membrane), and then dialyzed in the cold for 3 days against the 0.1 M Tris HCl, pH 8.

**Table I.** Recovery of Activity in the Unfolded Reduced Fragments of BSA upon Air Reoxidation

| Fragment assayed | % activity recovered |
|---|---|
| Refolded fragment A | 45 ± 5[a] |
| Refolded fragment B | 15 ± 2[a] |
| Refolded fragment A + refolded fragment B | 7 ± 2[b] |

[a] Recovered activity is calculated by assaying for catalytic activity after a refolded sample of one fragment is added to ca. an equimolar concentration of the complementary "native" fragment. This activity is then normalized (to give % activity recovered) by comparing it to the activity of the "native" sample in the presence of a similar equimolar concentration of the complementary "native" fragment. The entire reoxidation procedure was done twice and each reoxidized sample was assayed at least three times. [b] This activity is calculated relative to a control which contained both "native" A and "native" B at the same final concentrations (each ca. 10 μM) as was used for the refolded samples.

The extent of refolding of each isolated fragment was determined by assaying for catalytic activity in the presence of ca. an equimolar concentration of the complementary "native" fragment.[10] This was compared to a control experiment in which the equivalent "native" fragment was assayed for activity in the presence of the same concentration of its "native" complementary fragment. The results (Table I) indicate that both fragment A and fragment B can, in fact, refold independently in relatively good yields, when the catalytic activity of the products is used as a criterion for refolding.[11]

In these experiments in which the refolding milieu for a given fragment also contained the complementary "native" fragment, some aggregation was evident in that the solutions scattered light. Though this precluded a quantitative determination of the extent of refolding (the actual protein concentrations in these reconcentrated solutions are uncertain) these solutions also exhibited substantial activity (ca. 10–30% of full activity). It is also interesting to note that a moderate amount of activity was detected when refolded fragment A was added to refolded fragment B (Table I). This indicates that a significant fraction of both A and B must have refolded correctly, and that the observed regain of activity when a refolded fragment was combined with the complementary "native" component (see above) could not represent a "last minute" further refolding and reoxidation of the fragment due to the presence of the complementary "native" fragment. Within experimental error it appears that the combination of refolded fragment A with refolded fragment B may be random, as the observed relative activity of the product (ca. 7%) is only slightly greater than what would be expected if there were a random recombination of the catalytically active and inactive refolded fragments.

We have already shown that the catalytic activity of BSA is very sensitive to the conformation of the protein, and that it is particularly sensitive to the proper arrangement of disulfide bonds in the molecule.[8c] Thus it is reasonable that a very high degree of native structure is present in the refolded fragments. Considering that a random reoxidation of disulfide bonds in the two fragments can in principle generate greater than $10^6$ species for both B and A if the reoxidation of disulfide bonds is random,[12] the regeneration of a significant fraction of catalytic activity for the *recombined*, reoxidized fragments (and, by implication, therefore, the regeneration of considerable native-like structure) is truly remarkable and strongly supports the basic domain folding concepts enunciated by Wetlaufer.[3]

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## References and Notes


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(9) We will designate fragments as "native" if they were made from whole BSA and were *not* subjected to the reoxidation procedure.
(10) Final concentrations of each fragment were ca. 10 $\mu$M. Experiments in which a large molar excess of the complementary "native" fragment was used gave similar results.
(11) In control experiments the unfolded, reduced fragments were reacted with iodoacetate to prevent subsequent refolding and reoxidation after the urea and mercaptoethanol were dialyzed out. As expected, these permanently unfolded fragments had absolutely no catalytic activity, when assayed against each other or with the corresponding complementary native fragments.
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(13) Research Career Development Awardee of the National Institutes of Health, No. AI-00062-01.


**Ronald P. Taylor,*[13] Ann Silver**
*Department of Biochemistry, School of Medicine*
*University of Virginia*
*Charlottesville, Virginia 22901*
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## Decacarbonyltriosmium Complexes of Some Conjugated Dienes. The Crystal Structures of $Os_3(CO)_{10}$(*s-cis*-$C_4H_6$) and $Os_3(CO)_{10}$(*s-trans*-$C_4H_6$)[1]

*Sir:*

Examination of the coordination and mobility of ligands in metal cluster compounds provides detailed data that may be applicable to the less accessible problem of characterizing species chemisorbed on metal surfaces.[2] Metal cluster complexes of conjugated dienes are rare and have not been well-characterized structurally.[3] We wish to report the preparation of a set of decacarbonyltriosmium complexes of conjugated dienes, some NMR evidence concerning the preferred modes of coordination, and the crystal structures of isomeric complexes of the s-cis and s-trans forms of 1,3-butadiene.

The unsaturated metal cluster compound $H_2Os_3(CO)_{10}$ was found to react readily with excess diene at room temperature in cyclohexane solution. Chromatographic separation provided the major cluster product, of formula $Os_3(CO)_{10}$(diene)(diene = 1,3-butadiene (**1a**), 2-methyl-1,3-butadiene (**2**), 2,3-dimethyl-1,3-butadiene (**3**), *trans,trans*-2,4-hexadiene (**4**), and 1,3-cyclohexadiene (**5**)), as a stable yellow solid in each case.[4] With 1,3-butadiene an additional compound (**1b**) of the same formula was isolated in low yield (vide infra). The mass spectrum of each compound includes a molecular ion and ions due to loss of up to ten carbonyl ligands.[5]

The ambient temperature $^1$H NMR spectrum obtained for **1a** consists of three, well-separated multiplets ($\tau$ 4.74, 7.68, 9.51 in $CDCl_3$) assigned in order of increasing field to the s-vicinal protons ($H_c$, $H_{c'}$) and to the geminal protons respectively cis ($H_b$, $H_b$) and trans ($H_a$, $H_{a'}$) to $H_c$ or $H_{c'}$. The coupling constants determined by iterative simulation ($J_{ab}$ = 2.89, $J_{ac}$ = 8.58, $J_{ac'}$ = −0.79, $J_{bc}$ = 7.17, $J_{bc'}$ = 1.02, $J_{cc'}$ = 4.66 Hz) compare closely with those of the tricarbonyliron complex of *s-cis*-butadiene.[6] At low temperatures (−90°) each of the three multiplets splits into new signals ($\tau$ 3.90, 4.78, 5.17 (1:2:1); 7.32, 7.47, 7.90 (1:1:2); 9.16, 9.65 (2:2)), signifying the presence of two interconverting, almost equally populated conformers. One species is symmetrical, with respect to the two halves of the diene; the other is unsymmetrical. Ambient spectra for complexes **2**, **3**, and **5** are each also consistent with symmetrical coordination of the diene in the s-cis form.[7] At −95° the spectrum of **5** is split, indicating that an unsymmetrical species is present; a symmetrical conformer is not observable. In contrast, the spectra of **2** and **3** are unchanged to −80 and −124°, respectively, suggesting that these compounds adopt only (or predominantly) a symmetrical structure.[8]

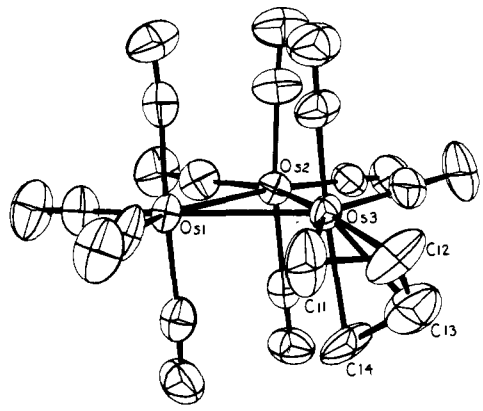


**Figure 1.** Molecular geometry of $Os_3(CO)_{10}$(*s-cis*-$C_4H_6$) (**1a**).

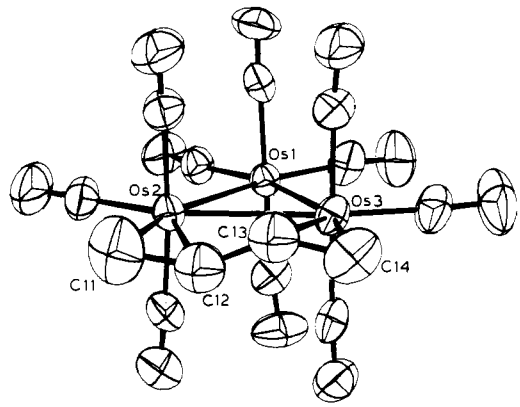


**Figure 2.** A view of the $Os_3(CO)_{10}$(*s-trans*-$C_4H_6$) (**1b**) molecule. Carbon atoms C(12) and C(13) are represented by isotropic thermal ellipsoids.

The solid-state structure of complex **1a** has been determined by x-ray diffraction. The complex crystallizes in space group $P2_1/n$ with a cell of refined dimensions $a$ = 8.051 (2) Å, $b$ = 14.778 (3) Å, $c$ = 15.356 (2) Å, and $\beta$ = 94.60°. Data were collected on a Syntex $P\bar{1}$ diffractometer using Mo K$\alpha$ radiation and corrected for absorption effects. The structure was solved by conventional Patterson and Fourier methods. Least-squares refinement of 2418 independent, observed reflections gave a conventional $R$ factor of 0.036. A view of the molecular geometry is shown in Figure 1. The structure is derived from that of $Os_3(CO)_{12}$[9] with substitution of an axial and an equatorial carbonyl at Os(3) by the *s-cis*-butadiene ligand. Carbon atoms C(11) and C(12) are nearly in the $Os_3$ plane, but C(13) and C(14) are pulled away substantially from the axial site due to the small bite angle of the diene. The osmium–axial olefin bond (Os(3)–C(13) = 2.24 (2), Os(3)–C(14) = 2.30 (1) Å) appears weaker than the osmium–equatorial olefin bond (Os(3)–C(11) = 2.24 (2), Os(3)–C(12) = 2.20 (1) Å). The Os(2)–Os(3) bond, trans to the equatorial olefin, is slightly longer (2.884