The Active Site Sulfhydryl of Aconitase Is Not Required for Catalytic Activity*

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Previous reports have demonstrated that aconitase has a single reactive sulfhydryl at or near the active site (Johnson, P. G., Waheed, A., Jones, L., Glaid, A. J., and Gawron, O. (1977) Biochem. Biophys. Res. Commun. 74, 384-389). On the basis of experiments with phenacyl bromide in which enzyme activity was abolished while substrate afforded protection, it was concluded that this group was an essential sulfhydryl. We have further examined the reactivity of this group and confirmed the result that, when reagents with bulky groups (e.g. N-ethylmaleimide or phenacyl bromide) modify the protein at the reactive sulfhydryl, activity is lost. However, when smaller groups, e.g. the SCH₃ from methylmethanethiosulfonate or the CH₂CONH₂ from iodoacetamide, are introduced, there is only partial (50%) or no loss of activity. Experiments were performed to obtain evidence that these reagents are modifying the same residue. Methylmethanethiosulfonate-treated enzyme showed an increase in the $K_\text{m}$ for citrate from 200 to 330 μM. EPR spectra were taken of the reduced N-ethylmaleimide- and iodoacetamide-modified enzyme in the presence of substrate. The former gave a spectrum typical of the substrate-free enzyme, while the spectrum of the latter was identical to enzyme with bound substrate. We, therefore, conclude that modification of this sulfhydryl affects activity by interfering with the binding of substrate to the active site and is not essential in the catalytic process.

Aconitase has been shown to contain a single reactive cysteine residue at or near the active site (2). Because the enzyme was inactivated by phenacyl bromide and protected by substrate, Johnson et al. (2) concluded that this SH is essential for enzyme activity. Ramsay et al. (3) demonstrated that the reactivity of this SH to either 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)¹ or N-ethylmaleimide (NEM) was identical whether inactive or active aconitase was used and that labeling had no effect on the EPR signal of the Fe-S cluster of the active enzyme. Dreyer et al. (4), using spin label techniques based on analogues of NEM and phenacyl bromide, have placed this SH group approximately 12 Å from the center of the Fe-S cluster which is now known to be involved in the catalytic process of the enzyme (5-7). In view of our present knowledge of aconitase as an Fe-S protein, the possibility had to be considered that this SH group might be released or engaged during cluster interconversions (8-12) or that it might serve as the base responsible for the direct transfer of H⁺ between citrate and isocitrate (13, 14).

Smith et al. (15) have demonstrated the utility of modifying sulfhydryls on enzymes with alkyl methanethiosulfonates of varying size to separate inactivation caused by blocking an essential catalytic group from that caused by steric hindrance of substrate binding. A study of pyruvate oxidase by Koland and Gennis (16) showed that, even though NEM completely inactivated the enzyme, methylmethanethiosulfonate (MMTS) was only partially inhibitory. This reagent, which reacts rapidly and specifically with SH groups in a reversible manner, introduces a much smaller group (SCH₃) into the protein than does NEM or phenacyl bromide. In light of this observation, we have examined the effects of a number of different SH-modifying reagents, including MMTS, on aconitase.

MATERIALS AND METHODS

NEM and iodoacetic acid were obtained from Schwarz BioResearch. The iodoacetic acid was recrystallized from ether/hexanol. MMTS, DTNB, and iodoacetamide (IAM) were obtained from Sigma. IAM was recrystallized from water. The preparation of aconitase and the sources of all other chemicals and enzymes were as described previously (9, 10).

Active enzyme free of activating reagents was prepared according to Ref. 9. All enzyme activities were determined by following the formation of aconitate from citrate (9) except in determining the $K_\text{m}$ of citrate for the MMTS-modified enzyme. In this case the coupled assay of Rose and O'Connell (13) was used.

Experiments were performed anaerobically when prolonged exposure of the substrate-free enzyme to air was involved, because oxidation of the Fe-S cluster and concomitant loss of enzymatic activity would make it difficult to obtain valid controls. Photoreduction of aconitase for EPR spectroscopy was done according to Ref. 10.

RESULTS

Reaction with NEM—Aconitase is rapidly inactivated when incubated with NEM under a variety of conditions. The result

¹ The abbreviations used are: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NEM, N-ethylmaleimide; MMTS, methylmethanethiosulfonate; DT, diithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; IAM, iodoacetamide.
of a typical experiment, e.g. incubation of 7 μM enzyme with 0.8 mM NEM, pH 7.5, at 0 °C shows complete inactivation within 15 min. When the experiment is repeated in the presence of 1 mM citrate, 90% of the original activity remains at 15 min.

Reaction with MMTS—To characterize further the possible catalytic role of the reactive sulphydryl, the reversible SH-specific reagent MMTS was tested. To prevent inactivation of the enzyme by oxidative damage to the Fe-S cluster, experiments and sampling were performed under anaerobic conditions. Although, as with NEM, the loss of activity was rapid, this loss was not complete (Fig. 1) and leveled at 50% of the initial activity.

Upon addition of dithiothreitol (DTT) virtually all the activity could be restored. When the experiment was repeated in the presence of substrate the rate of inactivation was too slow to be followed. However, when the reaction was carried out in the presence of 10 mM tricarballylate, it proceeded at a rate suitable for observation, approximately half that for the unprotected enzyme (Fig. 1). To determine that this SH group was identical to that reacting with NEM, experiments were performed where NEM was added after MMTS. There was no further loss of activity. This observation together with the results reported by Plank and Howard (17) demonstrates clearly that MMTS reacts with the same SH as NEM. Since introduction of the SCH₂ group into the protein does not abolish catalytic activity, the question now arises as to how this group affects the binding of substrate. Determination of the $k_\text{on}$ for citrate of the MMTS-modified aconitase gave a value of 330 μM compared to 200 μM for the unmodified enzyme. This is evidence that the decrease in activity is due to a weaker binding of substrate.

Reaction with IAM and Iodoacetate—Although previous work (18) had indicated that neither iodoacetate nor IAM inhibited enzyme activity, it seemed worthwhile repeating these experiments in view of our results with MMTS. In the present study the enzyme was incubated at pH 8.25 with IAM and at high reagent to enzyme concentration ratios in order to assure optimal reaction conditions (19). Under conditions where this ratio was greater than 1500:1, no loss of enzyme activity was observed over a 45-min period. That the sulphydryl group was blocked could be demonstrated by the subsequent addition of NEM to the reaction mixture which did not cause any loss of activity. Under these conditions the native enzyme would have been rapidly inactivated. Thus, it is clear that the reactive SH group is not a catalytic group and that, most likely, modification of this group alters binding of substrate as was evidenced by the higher $K_m$ for citrate for the MMTS-treated enzyme. An alternative method to examine this effect would be to look at the EPR signal of the [4Fe-4S] cluster of reduced active aconitase which is dramatically changed in the presence of substrates (10). There is tight binding of substrate to this form of aconitase as measured by the change in its EPR spectrum ($K_\text{D} = 1 \text{ μM}$). When the NEM- or IAM-treated enzymes are photoreduced in the presence of 1 mM substrates, the EPR spectra of the two samples differ (Fig. 2). The spectrum of the NEM-treated enzyme is identical to that of substrate-free enzyme, while the spectrum of the IAM-treated enzyme is that obtained when substrate is bound to the enzyme. Assays of the two samples before photoreduction demonstrated that the NEM-treated enzyme was inactive, while the IAM-treated enzyme was fully active and was protected against inactivation by NEM. It can be concluded from these results that the introduction of the bulky group from NEM blocks the binding of substrate to aconitase while the smaller acetamide group does not. It is also obvious from the EPR spectra that the presence of NEM bound to the reactive SH group leaves the Fe-S cluster intact.

Interestingly, iodoacetate in contrast to IAM does inactivate the enzyme, albeit considerably slower than NEM or MMTS. When 10 μM enzyme is incubated anaerobically at pH 8.0 with 9.1 mM iodoacetate, slow loss of activity occurs over a 3-h period with less than 10% remaining at this time. This suggests that the charge on the iodoacetate group affects

FIG. 1. Reaction of active aconitase with MMTS and its reversal by DTT. Aconitase (13 μM) in 0.1 M Hepes (pH 7.5) was incubated anaerobically at 20 °C with 100 μM MMTS, and activities were determined at the times indicated. At the arrow, 10 μl of 10 mM DTT, final concentration 250 μM, was added and activities were again determined (O). The reaction was repeated in the presence of 10 mM tricarballylate (●) to show the protective effect of the inhibitor.
both the rate of the reaction and subsequent binding of substrate. 

**Reaction with DTNB—DTNB (Ellman’s reagent) has proven to be a very useful and sensitive reagent for the quantitation of sulfhydryls in proteins (20).** The amino acid analysis of aconitase shows a total of 12 cysteine residues/mol of protein (21). To determine that only one of these sulfhydryls is fast reacting, a number of experiments with DTNB were performed. In Fig. 3 results are given where loss of activity and increase in absorbance at 412 nm are correlated for enzyme treated with DTNB in the presence and absence of substrate. In the absence of substrate there is a precipitous drop in activity with a concomitant rise in absorbance at 412 nm. There is no such loss of activity or increase in absorbance when citrate is present. When the data of specific activity versus quantity of SH reacting with DTNB are plotted, it is evident that complete loss of activity is associated with one fast reacting cysteine. The data of Fig. 4 provide strong evidence that this cysteine is identical to the one modified by IAM without loss of activity. Here, when the DTNB reaction of IAM-blocked and native enzyme are compared, the burst in absorbance at 412 nm is not observed for the blocked enzyme. The difference in absorbance for the normalized curves is equivalent to a difference of one SH reacting. The loss of activity of the blocked enzyme on exposure to DTNB was greater than is seen in many of the other experiments. As the experiment was performed aerobically, this is most likely due to oxidative damage of the cluster at this pH and temperature. When the reaction mixture with IAM-treated enzyme was kept at 0 °C there was very little loss of activity when DTNB was added.

The reaction rate of DTNB with aconitase varies not only with pH but is also sensitive to the type of buffer used. When 12.5 μM enzyme was allowed to react with 200 μM DTNB at pH 8.0 in the following buffers (all at 0.1 M): potassium phosphate, Tris sulfate, Tris chloride, and Hepes-K+, the percent activity remaining at 1 min was 73, 5, 6, and 36, respectively. It is probably not surprising that an enzyme which operates on trianionic substrates should be sensitive to multivalent buffer anions.

Gilbert (22) has proposed that glutathione may be a “third messenger,” which would modulate enzyme activity by thiol/disulfide exchange. To test the possibility of a regulatory role for the reactive sulfhydryl in accord with this hypothesis, aconitase was incubated with oxidized GSSG for prolonged periods of time. In one such experiment in which the ratio of GSSG to enzyme was 200:1, no loss of activity was observed within a 90-min interval. Thus, no evidence was obtained to support the role of glutathione as a modulator of aconitase activity.

It should be pointed out that, although the studies in this report were conducted with active enzyme, the inactive [3Fe-4S] form of aconitase (3) and even the apoenzyme also possess the single reactive cysteine residue. 

**DISCUSSION AND CONCLUSIONS**

Exposure of aconitase to a number of sulfhydryl-directed reagents including NEM, phenacyl bromide, and DTNB results in the rapid and complete loss of activity. However, when the enzyme is treated with other SH-reactive reagents, different results are obtained. Using MMTS we find the enzyme loses only 50% of its activity, while treatment with IAM results in no loss at all.

Experiments where the subsequent addition of NEM did not alter activity provide strong evidence that these reagents react with the same sulfhydryl. Reactions of DTNB with active and IAM-treated active enzyme confirmed earlier results that this is a single SH. Binding studies by EPR of the NEM- and IAM-treated enzyme indicated that binding of substrate (at concentrations 1000 times that of the apparent Kd for the unmodified enzyme) cannot occur when a bulky group is bound to the reactive sulfhydryl of the enzyme. Interference with binding is further evidenced by the increase in the Ke for citrate for the MMTS-modified enzyme from 200 to 330 μM. The size of the modifying group however is not the only factor affecting enzyme activity. Introduction of the relatively small acetate group, certainly not larger than the acetamide group, results in almost total loss of activity. Apparently the charge on the acetate group also impairs binding of substrate. These results lead us to conclude that the single reactive SH is not essential for catalytic activity, but that its modification may affect the binding of substrate to the active site. That the various SH reagents modify the same sulfhydryl is established by protein chemical methods as reported in Ref. 17. Experiments exploring the possibility
that this SH group might be a cluster ligand under certain circumstances are reported in an accompanying paper (12).

REFERENCES